# FURTHER RESEARCHES INTO INDUCED CELL-REPRODUCTION AND CANCER

H. C. ROSS

VOL. II.

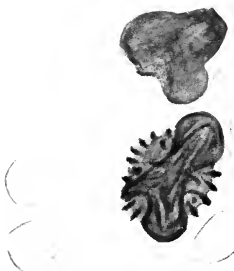
THE McFADDEN RESEARCHES

# FURTHER RESEARCHES

INTO

# INDUCED CELL-REPRODUCTION AND CANCER

VOL. II



More fixed and stained blood-films containing lymphocytes in the act of cell-division induced by anxeties continue to be made by the process described in *Further Researches into Induced Cell-Reproduction and Cancer*, Vol. I., page 34. The above illustrations are paintings from two of these fixed films. The upper one is of a lymphocyte in a stage resembling the anaphase and the lower one is of a lymphocyte in a stage resembling the telophase of mitosis · both profile aspect. All the illustrations in colour contained in this book were painted by Miss E. H. Barry.

[*Frontispiece*

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VOL. II

CONSISTING OF PAPERS

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WITH ILLUSTRATIONS

THE JOHN HOWARD
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# PREFACE

THE present volume is concerned with the elucidation of the theory that cell-proliferation and possibly cell-development are directly brought about by chemical agents set free by cell-death, and it is the third one published on this subject. The first book was published on December 15, 1910, under the title of *Induced Cell-Reproduction and Cancer*.[1] A sequel of the same researches appeared in September 1911, with the title of *Further Researches into Induced Cell-Reproduction and Cancer*,[1] and the present volume is a continuation. The first book described methods by which certain individual human cells can be made to divide in response to chemical agents, together with suggestions as to the association between this subject and the cancer problem. In the second volume the actual substances which cause the cell-divisions were enumerated and classified. In the present volume, however, experiments will be described which show how the previous work with individual cells has

[1] London, John Murray, and P. Blakiston's Sons & Co., Philadelphia.

been confirmed by experiments on animals; for we are now in a position to produce cell-proliferation and swellings resembling tumours in the living creatures by the action of the same chemical substances which induce individual cell-multiplication.

On June 1, 1911, the McFadden Researches and their original staff (which has been supplemented) were transferred from Liverpool to London, where, by the courtesy of the governing body of the Lister Institute of Preventive Medicine, some laboratories have for the time being been placed at our disposal. For this hospitality we desire to express our grateful appreciation.

H. C. Ross.

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# FURTHER RESEARCHES INTO INDUCED CELL-REPRODUCTION AND CANCER

## I

## ON THE EXPERIMENTAL PRODUCTION IN ANIMALS OF CELL-PROLIFERATION RESEMBLING BENIGN TUMOURS BY THE DIRECT INOCULATION OF AUXETICS

By H. C. Ross and J. W. Cropper

Since auxetics will cause the cell-division of individual cells *in vitro*, it is reasonable to suppose that, when these substances are inoculated into experimental animals in such a way that they come into prolonged contact with the tissue-cells, the latter must proliferate and ultimately produce some form of tumour. In the present chapter large numbers of these inoculations will be described together with the resulting tumours which appeared.

We may begin by giving a brief *résumé* of the researches which led up to these *in-vivo* experiments. About six years ago a new

method of vital staining was devised. Instead of having to examine cells which either were killed, fixed, and stained, or which were merely placed alive on a slide under the microscope, the cells were spread alive under a cover-glass on a film of jelly, which, if required, could contain the stain; and the cells absorbed the stain when in the living state. As shown in the former volumes, this method revealed many new facts about the cytology of certain cells, especially of blood-cells; for instead of the cells being fixed, and frequently distorted by manipulation, they were now seen alive with their component elements differentially stained in colours. By the manner in which the cells could be made to absorb the stain from the jelly, many points in connection with the diffusion of substances into living cells were elicited. In addition to providing vital staining, the jelly method—since other chemicals can be mixed with the jelly—also supplied a means of testing the actions of various chemicals on the living cells; and as some of the laws of the diffusion of substances into them had been found out, the cells could now be made at will to absorb the various substances contained in the jelly. While this was going on, the effect could be watched by means of the microscope, and at the same time the component parts of the individual cells, being brightly stained and differentiated, could be critically observed with the highest magnification. The majority of cells experimented with consisted of white blood-corpuscles from the

peripheral circulation, but, in addition, epithelial cells, nucleated red cells, and others were employed. To put it briefly (for all the details have been published in the former volumes), it was found that alkaloids excite amœboid movements in the cells, and subsequently the important fact was discovered that extracts of dead tissues induce the living cells to reproduce themselves under the microscope. The methods were elaborated and these facts further investigated; the actual chemical substances contained in the extracts of dead tissues were isolated, and facts were established which tended to show that normal individual cell-reproduction is brought about by the cells themselves absorbing the chemical agents set free as the result of cell-death. These agents were called *auxetics*.

Based on these experiments on individual cells, working hypotheses (published in the first volume, *Induced Cell-Reproduction and Cancer*) were elaborated as to the causes, firstly of normal cell-proliferation, secondly as to the cause of benign tumours, and thirdly as to the cause of cancerous tumours. We suggested that normal cell-multiplication was the result of gradual and physiological cell-death. Benign tumours, including the cell-proliferation of healing, we believed to be caused by more considerable localised cell-death, due in the first place to some injury and subsequently becoming more and more progressive; for it would appear that, after a certain point, the more the cells proliferated, the more cell-

death must be occasioned, and therefore in its turn the greater will the cell-proliferation be until healing sets in and the growth is limited by the production of connective tissue. The question of malignant cell-proliferation will be discussed in the next chapter.

Such were the facts and theories elicited by experiments with the jelly method. But by this method cells were merely experimented with *in vitro*; that is, when they were removed from the body and placed under the microscope. In order to prove the results of these experiments, however, it is obviously necessary to endeavour to see whether the same results can be produced *in vivo*; that is, when the cells are not removed from the body, but are living in their natural surroundings. Hence, having obtained as much evidence as possible with individual cells, we now proceeded to make a series of experiments consisting of the inoculation of the auxetics into animals.

For a complete list of auxetics the preceding volume may be consulted.[1] The auxetics may be divided into two groups—natural and artificial. All the natural auxetics are substances set free by cell-death. Several of them contain the amidine grouping, such as creatine and creatinine. Some of the primary aliphatic amines, such as methylamine, or some of the amino-acids, such as tyrosine, have also proved to be auxetics judging by the experiments

[1] *Further Researches into Induced Cell-Reproduction and Cancer*, Vol. I.

on the individual cells. The experiments on animals were made as follows:

MICE: *Natural auxetics.*—The animals first employed were mice; and the hypodermic needle was inserted into the axillæ in the hope of reaching the mammary tissue, but, as will be shown later, this was not successful. Aseptic precautions were always taken, and abscess occurred only in two cases. The first auxetic tried was guanidine hydrochloride, because it was one of the simplest natural amidines. It is so poisonous, however, that it was necessary at the outset to find the lethal dose of it for the animals.

TABLE I (MICE)

*To Find the Lethal Dose of Guanidine*

| Number of Animals | Number of Inoculations with each Animal | Strength of Solutions | Equivalent in Grammes | Result |
|---|---|---|---|---|
| 6 | 2 | 1% | 0·002 | Nil |
| 2 | 1 | 2% | 0·004 | Nil |
| 3 | 1, 2, 2 | 2·5% | 0·005 | 1 died |
| 3 | 1 | 5% | 0·010 | 1 died |
| 4 | 1 | 10% | 0·020 | 3 died |

Guanidine causes convulsions. It begins to affect mice visibly when 0·005 gramme is inoculated, and 0·02 gramme is lethal. Therefore its application for these experiments is limited; and, judging by the way in which the fluid soon disperses, it seems to remain locally in the tissues for a very short time. Hence little or no result was obtained with it.

TABLE II (MICE)[1]

*Natural Auxetics*

| Number of Animals | Number of Inoculations with each Animal | Strength of Solutions | Equivalent in Grammes | Result |
|---|---|---|---|---|
| | | Guanidine (amidine) | | |
| 6 | 10 | 1% | 0·002 | Slight "thickening" at site of inoculation |
| | | Creatine (amidine) | | |
| 3 | 6 | 5% | 0·010 | Nil |
| | | Leucine (amino-acid) | | |
| 3 | 9 | 1% | 0·002 | "Thickening" |
| | | Tyrosine (amino-acid) | | |
| 2 | 14 | 1% | 0·002 | Nodules appeared in 10 days |

Creatine, leucine, and tyrosine were then tried. Practically no result was obtained with creatine, a little thickening occurred in response to leucine, but distinct nodules appeared in a week at the site where tyrosine was injected, the inoculations being repeated daily (Table II).

*Artificial auxetics.*—With the artificial auxetics, however, better results were obtained, for a saturated watery extract of blast-furnace tar caused thickening, isobutylamine produced in a week a distinct tumour which ultimately broke down and ulcerated, and ethylamine had a very similar result.

[1] In all the experiments with mice inoculations were made daily in 3-minim doses.

TABLE III (MICE)

*Artificial Auxetics*

| Number of Animals | Number of Inoculations with each Animal | Strength of Solutions | Equivalent in Grammes | Result |
|---|---|---|---|---|
| | | Blast-furnace tar | | |
| 3 | 9 | Saturated watery extract | — | "Thickening" |
| | | Isobutylamine[1] | | |
| 3 | 10 | 1% | 0·002 | Tumour and ulcerate ulceration |
| 1 | 6 | 5% | 0·010 | |
| | | Ethylamine | | |
| 1 | 3 | 5% | 0·010 | Marked induration and ulceration |

TABLE IV (MICE)

*Controls*

| Number of Animals | Number of Inoculations with each Animal | Strength of Solutions | Equivalent in Grammes | Result |
|---|---|---|---|---|
| | | "Irritation" of hypodermic needle | | |
| 5 | 8, 8, 8, 4, 4 | — | — | Nil |
| | | Distilled water | | |
| 6 | 12, 12, 12, 12, 12, 12 | — | — | Slight "thickening" in two cases |
| | | Acridine | | |
| 4 | 4, 8, 10, 10 | 1% | 0·002 | Slight ulceration, which did not get worse, occurred in the animal inoculated 4 times |

[1] This experiment was unsatisfactory owing to the volatile nature of the isobutylamine, which became varied in strength when sterilised.

*Control experiments.*—Some preliminary control experiments were then made to determine whether similar thickening or tumours could not be induced merely by the puncture of the hypodermic needle or by the injection of distilled water (Table IV). The puncture of the needle had no effect at all, however, and the repeated injection of distilled water had a similar result except in two animals (out of six), in which slight thickening occurred at the site of inoculation, probably owing to the chronic injury.

The experiments with mice were rather unsatisfactory because these animals are small and delicate. Moreover, their mammary tissue is very thin, and is adherent to the skin, which renders it very difficult if not impossible to make certain that the liquid injected remains in contact with the mammary cells. The sections which were cut of the tumours produced showed that, while marked cell-proliferation was induced in each case by the auxetics, the cells which proliferated were connective tissue-cells and not those of the mammary gland, none of which appeared in the sections at all. The tumours, therefore, were of the nature of granulomata and fibromata.

Guinea-pigs: *Natural auxetics.*—As the experiments with the mice were unsatisfactory, the inoculations were repeated and some of them were elaborated, using guinea-pigs; and much better results were obtained. As before, the inoculations were made in the neighbourhood of the nipple. The natural auxetics were

first tried. Guanidine produced a small tumour, but little result was obtained by the inoculation of creatine. Tyrosine, on the other hand, invariably produced large tumours, which began to appear on the third day. Indeed, tyrosine seems to be the most satisfactory natural auxetic with which to induce cell-proliferation *in vivo*, for its action is most constant. The substance which has been called "globin," *i.e.* the filtrate of a solution of hæmoglobin precipitated by heat, also produced large granulomata, as did a solution of hæmoglobin, though to a smaller extent.

TABLE V (GUINEA-PIGS)[1]

*Natural Auxetics*

| Number of Animals | Number of Inoculations with each Animal | Strength of Solutions | Equivalent in Grammes | Result |
|---|---|---|---|---|
| | | Guanidine | | |
| 1 | 14 | 5% | 0·037 | Small tumour |
| | | Creatine | | |
| 1 | 9 | 2% | 0·015 | Nil |
| | | Tyrosine | | |
| 5 | 27, 24, 2, 19, 8 | 3% | 0·022 | Tumours in every case |
| | | "Globin" | | |
| 3 | 11, 6, 5 | 4% | 0·030 | Large tumours |
| | | Hæmoglobin | | |
| 1 | 12 | — | — | Small tumours |

*Artificial auxetics.*—These also produced tu-

[1] In all the experiments with guinea-pigs the inoculations were made daily in 10-12 minim doses.

mours at the seat of inoculation, theobromine and blast-furnace tar being the best of them.

TABLE VI (GUINEA-PIGS)

*Artificial Auxetics*

| Number of Animals | Number of Inoculations with each Animal | Strength of Solutions | Equivalent in Grammes | Result |
|---|---|---|---|---|
| | | Theobromine | | |
| 2 | 31, 9 | 3% | 0·022 | Tumours |
| 2 | 19, 2 | 5% | 0·037 | Tumours |
| | | Watery extract of blast-furnace tar | | |
| 1 | 19 | Saturated extract | — | Small tumours |

Auxetics, therefore, do undoubtedly cause cell-proliferation when the cells are in their natural surroundings and some of them, especially the natural one tyrosine, and the artificial one theobromine, will produce such large, hard, almost pedunculated swellings with unfailing regularity that we think that they can be rightly called tumours. The size of the tumours produced—that is to say, the extent of the cell-proliferation—up to a certain point varies directly with the quantity of auxetic inoculated. Some auxetics appear to be more powerful than others, although to what this is due we have no definite proof, but several factors must be taken into consideration. For instance, as shown by the *in-vitro* experiments, the diffusion of the substances into the cells is of great importance; and all the substances seem to have different "units of diffusion." [1] Again,

[1] See a paper by one of us in the *Proceedings of the Royal Society*, B, Vol. 81, 1909.

the diffusibility of the substances in the tissues into which they have been inoculated must have considerable effect on the size of the tumours produced, for if the substance is quickly removed from the site of inoculation, the proliferation must cease; and some of the auxetics seem to remain distending the tissue longer than others. Moreover, if the substance is less soluble and remains present in the tissues in the shape of a powder, it will have a greater effect, because there is, so to speak, a constant supply of it for the cells to absorb. On the other hand, if the substance is very soluble, it may be quickly excreted, when its effect can no longer be brought about.

If we now sum up the results of these *in-vivo* experiments, we think that they undoubtedly confirm the *in-vitro* experiments on the individual living cells. With the exception of creatine, which is not very effective, possibly on account of its being decomposed into urea, or owing to its diffusibility in the tissues, all the other auxetics tried invariably produced cell-proliferation, and some of them caused discrete, freely movable, tumours. No result in any way comparable to this was obtained by other substances which will not induce individual cell-division under the microscope. It is true that distilled water when inoculated will sometimes produce a little "thickening," but the comparison between this induration and the tumours induced by auxetics leaves no room for doubt about the reproductive action of the latter. The *in-vivo* experiments,

taken in conjunction with the fact that so far it has only been these auxetic substances which will directly induce cell-division on the jellies *in vitro*, appears to us to make it certain that they do immediately cause cell-proliferation.

As far as can be seen, the only possible fallacy is one based on what we consider to be an old misunderstanding. Many persons on seeing these induced tumours asked whether they were not merely produced by the " irritation " of the chemicals inoculated, in spite of the fact that the natural auxetics cannot be said to be " irritants," in the commonly accepted meaning of the word. It is well known that certain substances which have been called " irritants " do produce cell-proliferation or even small tumours, and the question arose whether it was not merely this effect which gave rise to the tumours in our experiments.

To the clinician the word " irritation " has a wealth of meaning, but to the cytologist, who considers the tissues from the point of view that they consist of groups of minute individual living cells, its meaning is not quite so clear. One can readily understand that if we place a powerful so-called irritant such as iodine or acridine on to the skin, a mucous surface, or in fact any tissue, violent " irritation " is set up ; and the same thing can be said about the so-called mechanical irritants. At first we begin to " scratch " the site, because the nerve terminations are stimulated, and tingling or even pain, which are degrees of the same thing, are caused. Inflammation,

which is another rather vague term to the cytologist, may ensue, attended by cell-proliferation, although hitherto no one has known what makes the cells proliferate. Ultimately ulceration may occur with destruction of tissue, in which case there is more cell-proliferation than ever round the edges.

The cytologist, however, asks himself what is the effect of the irritant on the individual cell. If the irritant causes cell-proliferation, how does it make the individual cells multiply ? A tissue largely consists of these cells, which, in their turn, consist of a cell-wall surrounding living protoplasm ; and it is difficult to imagine how a cell can be irritated by a chemical or by a mechanical force. The cells themselves are extremely delicate ; and we know by experiments on them *in vitro* that if they are subjected to the slightest mechanical force or to the action of irritating or in fact many artificial substances, all that happens is that they die. Individual cells subjected to a rapid poison or to pressure of any sort are never seen to reproduce themselves ; on the contrary, every precaution has to be taken to protect them from injury, either mechanical or chemical.

In all probability the so-called irritants cause local death. There is evidence of this in the desquamation which takes place after they have been applied to the skin ; and the chemical irritants appear merely to be such because they are poisons—and we speak of a force as an irritant only when it is strong enough

to cause some cell-death. Presumably any artificial (the term is used in accordance with the classification of auxetics) substance introduced into the body is a poison to some of the cells if it is introduced in sufficient quantity. Some substances are more poisonous than others; that is to say, some of them, weight for weight, will kill protoplasm more rapidly than others. The stronger when applied to a tissue are known as caustics, the weaker as irritants; the intrinsic result of both is probably the same, except that the former may cause destruction so rapidly that repair cannot keep up with it, while the latter may cause injury which is healed up almost as quickly as it occurs. It would appear, therefore, that the word "irritation" from a cytological point of view really means cell-death.

Mechanical irritants may come into the same category. A mechanical force is either sufficient to cause cell-death or it is not. If it is sufficient to cause rapid and extensive death, injury is occasioned; if only localised death takes place, "irritation" is the word used. The whole question of 'irritants" appears to be one of local life or death; that is, whether the individual cells will survive or not. It is difficult to imagine how there can be a half-measure.

Since the word "irritation" seems to imply cell-death, and since cell-proliferation occurs in sites which are subjected to irritation, cell-proliferation must in these instances be directly associated with cell-death. But all the previous work with individual cells under the microscope

has clearly shown that the products of cell-death are the direct causes of cell-division, and it is by the direct inoculation of these products that the tumours are produced. The natural auxetics, such as tyrosine and creatine, are not irritants, for they are physiologically produced in the body by cell-death—they are the result of irritation, not the cause of it—and individual cells will live for a long time in their presence under the microscope. Yet they produce cell-division *in vitro*, and tumours *in vivo*.

Some control inoculations were made, however, to try to settle this question of "irritants" experimentally. If these substances merely caused proliferation by virtue of some form of irritation, and not by the fact that they are the products of cell-death and therefore the actual causes of cell-division, the well-known irritants should cause far greater proliferation. The repeated irritation of a hypodermic needle in the tissue ought to produce as large a tumour as the auxetics do; but this was not the case. The argument might be raised that the tumours were due to the mechanical irritation of the fluids inoculated, but the inoculation of distilled water did not produce a tumour. The inoculation of a well-known and powerful irritant—acridine—had no effect whatever in producing a tumour, employing the word "tumour" in comparison with those induced by the auxetics. And the mechanical irritation of powdered glass introduced into the tissues merely produced a little induration, not the

large, discrete, freely movable swellings caused by the auxetics themselves. Insoluble salts, such as calcium oxalate and barium sulphate, remained as such at the seat of inoculation. They caused a little inflammation round the site, but no tumour.

TABLE VII (GUINEA-PIGS)

*Controls*

| Number of Animals | Number of Inoculations with each Animal | Strength of Solutions | Equivalent in Grammes | Result |
|---|---|---|---|---|
| | | "Irritation" of hypodermic needle | | |
| 4 | 10, 10, 8, 8 | — | — | Nil |
| | | Distilled water | | |
| 4 | 10, 10, 8, 8 | — | — | Slight induration occurred in one case |
| | | Powdered glass | | |
| 1 | 2 | — | — | Nil |
| | | Insoluble salt (calcium oxalate) | | |
| 1 | 7 | 5% suspension | — | Swelling composed of the salt |
| | | Insoluble salt (barium sulphate) | | |
| 1 | 7 | 5% suspension | — | Swelling composed of the salt |

Taking it all into consideration, therefore, we submit that the evidence may be taken as conclusive that cell-proliferation is brought about by the auxetic produced by cell-death, and, if the cell-death becomes "chronic," tumour may be produced.

But we cannot yet assert that this is the only cause of normal tissue-cell proliferation. There may be other exciting causes about which we know nothing, although, judging by former experiences with other biological examples, this is not probable. Take the case of the propagation of the parasite of malaria by means of the mosquito, or the propagation of Malta fever by goats' milk. For years many people, while admitting that the mosquito distributed one disease and goats' milk the other—for these facts were proved—still believed that in addition there were other methods of propagation. They suggested, for instance, that malaria parasites also bred in marsh water, and that the *Micrococcus melitensis* was also conveyed through the air or by insanitary conditions. None of these theories have been disproved, but they are slowly becoming forgotten in the face of the overwhelming importance of the methods of propagation which are absolutely proved to be right. Biological functions seem to have only one immediate cause; and when this is found out, although for some years a few people tenaciously maintain the old theories, gradually it becomes apparent that the one established cause will meet all the eventualities, and the older ideas gradually vanish.

It is quite possible that a similar condition will be found to exist with the cause of cell-proliferation. We now know that the products of cytolysis are *a* cause of cell-proliferation, but we do not know for certain that they are

the *only* cause of it. Probably they are, because it seems unlikely that a complex function like cell-reproduction should have more than one direct cause; and we consider that the weight of evidence is sufficient for it to be stated that the cell-proliferation of healing and benign tumours are immediately brought about in the manner described, in spite of the fact that, owing to experimental difficulties, we have not been able to produce tumours in every form of tissue in which they naturally occur.

## II

# ON THE EXPERIMENTAL PRODUCTION IN ANIMALS OF EPITHELIAL CELL-PROLIFERATION AND INFILTRATION BY THE DIRECT INOCULATION OF A MIXTURE OF AUXETICS AND AUGMENTORS

By H. C. Ross and J. W. Cropper

Malignant cell-proliferation, according to the working hypothesis based on the experimentation with individual cells *in vitro*, is considered to be due to a combination of two factors: there must be chronic local injury, with the production of auxetics causing prolonged normal cell-proliferation; and also we believe that certain substances which have been called augmentors must simultaneously be absorbed by the cells. In other words, the theory is that cancerous proliferation is immediately brought about by the causes of normal cell-proliferation (auxetics) *plus augmentors*. This theory has now been tested *in vivo*, and in the present chapter the results of the inoculation of combinations of auxetics and augmentors into animals will be described.

The jelly method of *in-vitro* staining showed that certain substances, especially alkaloids,

will excite amœboid movements in white blood-cells, and these substances have been called *kinetics*. In addition to this, while they do not themselves cause cell-division, some of these kinetics greatly augment the action of the auxetics in doing so. These were known as *augmentors*.

How this augmentation is produced by these chemicals we have no opinion to offer, but the action is very strikingly demonstrated by the jelly method. For instance, a jelly may be employed which, although it contains auxetic, does not contain it in sufficient quantity for cell-division to be induced with it under the experimental conditions. If a small quantity of an augmentor is now added to the jelly, advanced cell-division can easily be induced with it, although had the jelly contained no auxetic at all, no cell-division would have been induced, for the augmentors by themselves are not effective.

Further investigation of the action of these augmentors revealed the fact that some of them will augment the action of auxetics as much as fivefold; that is to say, if a given jelly contains only one-fifth of the minimum quantity of auxetic required to induce cell-division in a certain time, when the augmentor is added its efficiency to induce the division-figures in a certain time is restored. This chemical augmentation of the causes of normal cell-proliferation is a very striking fact in view of the increased proliferation which occurs in malignant growths, and what appears to

us also to be an important point is that the division-figures induced in lymphocytes by the mixture of auxetics and augmentors are frequently of the asymmetrical type resembling those seen in epithelial cells in carcinomatous growths.

A complete list of the known kinetics and augmentors will be found in the preceding volume; they have been divided into two groups—natural and artificial. In the experiments to be described, however, we are only concerned with the so-called natural ones—namely, those which are found in the tissues. Although there may be many of these natural augmentors, at present we only know of two—choline and cadaverine. These substances are known to be produced under certain conditions in a chronically injured site by the action of some forms of bacteria; and based on all these facts the working hypothesis as to the causation of malignant proliferation was arranged—an hypothesis which will be found set out at length in our first volume, together with the manner in which its details harmonise with the well-known symptoms and signs of cancerous growths.

Working on similar lines to the *in-vivo* experiments with auxetics described in the preceding chapter, the first animals employed were mice. Efforts were made throughout these experiments to make the auxetics and augmentors remain in contact with epithelial cells, but this has been very difficult. The mammary tissues of mice, as already mentioned, are

very thin, and proved to be unsuitable for this form of inoculation. Guinea-pigs were not much better, although in both animals epithelial cell-proliferation and infiltration were induced by causing ulceration, and the proliferation and infiltration occurred in the squamous epithelium of the skin. The experiments were made as follows:

MICE: *Augmentors by themselves.*—Although the augmentors do not by themselves induce cell-division *in vitro*, nevertheless this point was also tested in the first place on animals so as to control the experiments made under the microscope, but the augmentors by themselves had no effect in producing cell-proliferation.

TABLE I (MICE)[1]

*Natural Augmentors*

| Number of Animals | Number of Inoculations with each Animal | Strength of Solutions | Equivalent in Grammes | Result |
|---|---|---|---|---|
| | | Cadaverine | | |
| 3 | 10 | 1% | 0·002 | Nil |
| 1 | 2 | 5% | 0·010 | Nil |
| | | Choline | | |
| 5 | 2, 6 | 5% | 0·010 | Nil (3 died) |

MICE: *Mixture of auxetics and augmentors.*—When they were mixed with auxetics, however, better results were obtained. Mixtures of creatine and choline, or creatine and cadaverine, produced

[1] In all the experiments with mice the inoculations were made daily, using 3-minim doses.

only induration, tyrosine and choline caused nodules, and isobutylamine and cadaverine produced induration in two days, a distinct tumour in three days, and extensive ulceration in five days.

TABLE II (MICE)

*Mixture of Auxetics and Augmentors*

| Number of Animals | Number of Inoculations with each Animal | Strength of Solutions | Equivalent in Grammes | Result |
|---|---|---|---|---|
| | | Creatine and cadaverine | | |
| 2 | 7 | 1% of each | 0·002 of each | Nil |
| | | Creatine and choline | | |
| 2 | 5 | 1% of each | 0·002 of each | Nil |
| | | Tyrosine and choline | | |
| 3 | 10 | 1% of each | 0·002 of each | Nodules |
| | | Isobutylamine (artificial) and cadaverine | | |
| 6 | 10, 10, 10 8, 6, 6 | 1% of each | 0·002 of each | Thickening in 2 days, tumour in 3 days, and ulceration in 5 days |

GUINEA-PIGS: *Mixture of auxetics and augmentors.* The inoculation of a natural auxetic such as tyrosine and an augmentor such as cadaverine produces the very rapid appearance of a tumour. Indeed, in every experiment, the growth was so rapid and large that these experiments fully confirmed the results obtained *in vitro* on individual cells, which clearly showed that augmentors greatly increase the action of auxetics. Using a combination of

choline or cadaverine with isobutylamine, the tumours broke down and ulcerated; and in the sections of the edges of the ulcers the epithelial cells can be seen proliferating and infiltrating into the deeper tissues, resembling the same phenomena seen in epitheliomata. A very similar result was obtained with a watery " extract " of gasworks tar.

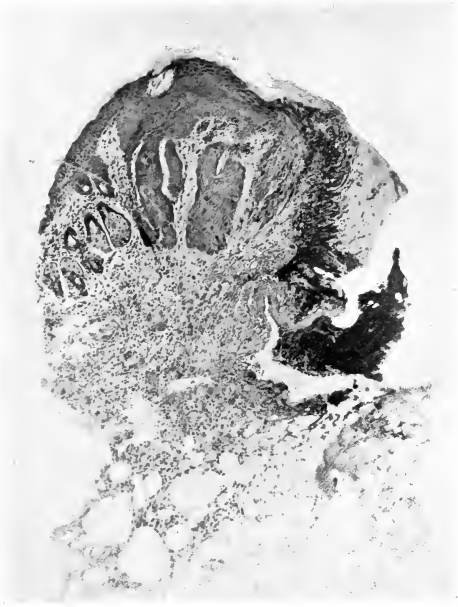
TABLE III (GUINEA-PIGS)[1]

*Mixture of Auxetics and Augmentors*

| Number of Animals | Number of Inoculations with each Animal | Strength of Solutions | Equivalent in Grammes | Result |
|---|---|---|---|---|
| | | Tyrosine and cadaverine (natural) | | |
| 3 | 11, 13, 7 | 3% and 1% | 0·022 and 0·007 | Tumour |
| | | Isobutylamine (artificial) and choline | | |
| 1 | 9 | 1% of both | 0·007 of both | Tumours |
| 3 | 3, 8, 8 | 5% and 1% | 0·037 and 0·007 | Tumour and ulcer |
| | | Isobutylamine (artificial) and cadaverine | | |
| 1 | 1 | 1% of both | 0·007 of both | Ulcer |
| 1 | 7 | 5% and 1% | 0·037 and 0·007 | Died |
| | | Gasworks tar | | |
| 2 | 26, 12 | Saturated watery extract | — | Tumours |

It would seem therefore that the experiments have demonstrated that conditions resembling the appearances of malignant epithelial cell-proliferation and infiltration can be produced by the inoculation of combinations of auxetics

[1] In all the experiments with guinea-pigs the inoculations were made daily, using 10-minim doses.



Section from the edge of an ulcerated tumour produced by the inoculation into an animal of a mixture of auxetics and augmentors.

and augmentors. By the sections it is difficult to distinguish between this artificially induced proliferation and that seen to occur in natural epithelioma. But it should be remembered that in natural epithelioma there are more factors to be taken into consideration besides the local malignant cell-proliferation and infiltration. The constant extension of the growth, the metastases, and the ultimate death of the victim are factors which are nearly always present. In our opinion all of them must be reproduced in animals *de novo* before one can say definitely that the cause of cancer is known. Still, one can now to some extent produce the local epitheliomatous condition, and we do not think that it will be long before the other conditions have been fulfilled. It is probable that they would have been already fulfilled had it not been for two difficulties—the one that it is not easy to obtain in animals an epithelial tissue suitable for experiment, and the other that up to a short time ago the tumours produced did not last long; for, depending as they did on the hypodermic syringe for their supply of auxetic, when the inoculations ceased the tumours gradually disappeared. It is to be hoped that the first difficulty may be overcome by employing the mammary gland of the goat. The second difficulty seems also now to be on the way of being removed by mixing alkali with the auxetics. Investigating by *in-vivo* experiments the laws of the diffusion of substances into cells as elicited *in vitro* by the jelly method, it has recently been found that

if the auxetics are rendered alkaline before they are inoculated, not only is the appearance of the tumours much more rapid, but they no longer tend to disappear when the inoculations cease. Sometimes the tumours remain

TABLE IV (GUINEA-PIGS)

*Mixture of Auxetics and Alkali*

| Number of Animals | Number of Inoculations with each Animal | Strength of Solutions | Equivalent in Grammes | Result |
|---|---|---|---|---|
| | | Tyrosine and sodium bicarbonate | | |
| 1 | 8 | 3% and 5% | 0·002 and 0·037 | Tumour produced in 48 hours |
| | | Theobromine and sodium bicarbonate | | |
| 1 | 8 | 3% and 5% | 0·022 and 0·037 | Rapidly induced tumour (died) |
| 1 | 6 | 3% and 5% | 0·022 and 0·037 | Very small tumour |
| 1 | 6 | 3% and 1% | 0·022 and 0·007 | Larger tumour |
| 1 | 4 | 3% and 2·5% | 0·022 and 0·018 | Tumour appearing in 48 hours, but lasting for 3 weeks |

for several weeks. These appear to be steps in the right direction, for one is now able to produce what may almost be called permanent cell-proliferation; and we hope that, instead of always having to make the tumours continue by the constant use of the hypodermic needle, two or three inoculations will produce tumours which can be observed for some time. Further, instead of inoculating the *augmentors* as we have been doing up to the present, we also

hope to be able to produce them in permanent benign tumours by introducing the organisms which manufacture them naturally in the tissues. In any case, it is now known for certain that the combination of auxetics and augmentors does produce—not actually epithelioma in the common acceptance of the term—but epitheliomatous cell-proliferation and infiltration. It is also known for certain that the auxetics are set free during chronic injury (irritation), and that the augmentors can be produced in a chronically injured site by the action of bacteria.

It may be mentioned that several years ago Leo Loeb produced epithelial infiltration by means of certain aromatic amines; and Drs. Stoeber and Wacker have also induced non-typical squamous epithelial growth by protein putrefactive products (*Muenchener Medizinische Wochenschrift*, May 3, 1910).

## III

# A COMPARISON BETWEEN THE DIVISION FIGURES INDUCED IN LYMPHOCYTES BY AUXETICS WITH THE JELLY METHOD AND THE MITOTIC FIGURES SEEN IN THESE AND OTHER CELLS IN SECTIONS OF TISSUES BY THE OLDER METHODS[1]

By H. C. Ross

At the meeting of the Pathological Section of the Royal Society of Medicine, held at the Lister Institute on November 7, 1911, a demonstration was given by us of the fixed specimens, photographs, and of a few stained living specimens of lymphocytes in the act of cell-division induced by auxetics.[2] A paper[3] on this subject was read the same evening by Sir Ronald Ross, K.C.B., F.R.S., in which he described the division figures as he had seen

[1] A paper read before the Royal Society of Medicine on March 19, 1912.

[2] A detailed description of this work will be found in the two earlier books.

[3] Published in *The Proceedings of the Royal Society of Medicine*, December 1911, and also in *Nature*, December 14, 1911 (abstract).

them both in the living state (demonstrated to him by us) and in the fixed films. He pointed out that there is strong evidence to show that the figures were those of cell-division, and stated that in his opinion there is no question that white blood-cells can be made to divide in response to auxetics. At the subsequent discussion, some gentlemen intimated that from what they had been shown they were not convinced that the figures were those of cell-division, because the latter were not exactly like the mitotic figures with which they were conversant from the observation of mitosis in fixed sections of tissues. These gentlemen, of course, have not had the opportunity of making a prolonged study of the induced divisions in cells, stained alive by the jelly method, as we have. To Sir Ronald Ross, on the other hand, we have had the privilege of demonstrating a considerable number of living division figures, in addition to submitting to him all the fixed films of them in our possession, and therefore he was in a position not only to discuss the *pros* and *cons* of the question, but also to contrast what he had recently seen with the normal resting blood cells with which he was so well acquainted by his many years' work with the parasite of malaria. But even Sir Ronald Ross has not had the opportunity to see as many of the division figures in all their phases as I have seen, and for this reason I now propose to discuss in more detail the comparison between the induced figures in lymphocytes and the appearance of cells found in the act of division by the older methods.

I may add that the same objection has been raised in America and elsewhere.

At the outset attention may be drawn to the following points. Hitherto the phenomenon of mitosis has been studied almost entirely by the observation of cells found in the act of division in sections of tissues which have been fixed and stained by the usual processes. Until divisions were induced in lymphocytes by the jelly method, individual cells have not been watched going through the phenomenon of mitosis from start to finish, with their morphological elements distinguished by differential staining. Cells have been seen in the resting stage, and cells have been "caught" in the various stages of mitosis; comparisons have been made between the various stages, and between the stages and the resting cell, and it has been *deduced* that this morphological element in the several phases is derived from that morphological element in the resting cell, and so on. These deductions are undoubtedly correct for the majority of cells, and, owing to this fact, hard and fast rules have been made; indeed, these rules have become so firmly established that, unless one can show that the induced figures which we assert to be mitotic figures conform exactly to the accepted pictures, critics are inclined to dispute that we are dealing with mitosis at all. It is important to realise that, while many classes of cell appear to be identical morphologically, we have no right to accept the statement that a certain structure in a given class of cell is necessarily

the same structure in another class of cell, merely because it looks the same and appears to have the same relations to other structures. As I have said before, I do not think that we are justified in taking for granted the nature of cellular elements until we have witnessed the whole life-history of the cell in question. Undoubtedly, in the main principles, when they divide by mitosis, cells seem to adopt a common method of reproducing themselves, but in the details of the division figures differences are frequently apparent. In cytology, especially in the cytology of blood-cells, speculations have been rife, sometimes emanating from the highest sources, and in many instances they have been universally accepted without a murmur, as such theories appear to meet the case.

Another point is that, owing to the usual teaching of cytology, one is apt to get into the habit of thinking that cells when they divide do so in definite stages or phases, into which, for convenience of description, the phenomenon of mitosis has been divided. It is not many years ago that in many cases the only details about cytology which students were taught were brief descriptions of the aster and disaster pictures of karyokinesis. The well-known diagrams of these stages were shown them, and I have actually found that some persons, when we are inducing cell-division, seem to expect one to make a living dividing cell always resemble one or other of these diagrams. Cells do not conveniently dwell in the prophases, metaphases, anaphases, or telophases. Mitosis

is largely the phenomenon of the "shuffle" of the chromatin, so that each daughter cell may receive a due share of the hereditary characteristics; it is a sedate process which goes on steadily without any pause during cell-division. In living cells one frequently sees this shuffle taking place satisfactorily, although the figures appear to be atypical. The so-called phases of mitosis are arbitrary, arranged by cytologists. They are very convenient, and I believe that they have been so arranged because, by the fixation methods, large numbers of cells have happened to be found in the act of presenting one or other of them. When one is inducing cell-division, however, one does not necessarily have the same fortune, and it is the exception rather than the rule to see cells presenting one or other of the well-known phases, which nowadays are nearly always only diagrammatically represented in books on cytology. It is a matter of chance whether one sees cells in this or that well-known phase. In reality the probabilities are against it, and if a section of a tissue is looked at critically one may frequently see more cells in a transitional stage between two of the so-called phases than actually in the phases themselves. Unfortunately, it is not common for specimens to be examined thus critically; one usually examines a section of a tissue with a comparatively low power, casually passing field after field in front of the objective. If a *typical* mitotic figure appears, it immediately arrests attention because it is so familiar, but figures not in the well-known phases are apt

to be passed over. Hence people become inclined to consider that unless cells present some conventional picture of mitosis, the latter are not undergoing the phenomenon of indirect cell-division.

The question of trying to convince people, which was raised at the meeting, is almost entirely a question of being able to produce records of one's work. Other people, of course, want to see the division figures for themselves, and this has proved to be the most difficult part of our researches. In order to do it there are three courses open at present. One is to repeat the experiments in the presence of others, and to try to get some good specimens of division figures in the living cells. Another is to take photographs of the living cells in the act of division (which we did, and for reasons already given [1] proved to be most unsatisfactory); and the third method is to fix some specimens of the dividing cells in the way described in our last publication,[2] and to show the fixed films. I have already described in both of the former publications the difficulties which attend the demonstration of the divisions in living cells to large numbers of people. The cells do not live long, and soon become achromatic. Several fields may have to be searched through before good specimens are found, which involves delay and perhaps disappointment. Even the living cells frequently appear distorted owing to the fact that they are pressed out between the jelly and cover-glass, and until one

[1] *Induced Cell-Reproduction and Cancer.*

[2] *Further Researches into Induced Cell-Reproduction and Cancer.*

happens to have seen an undistorted specimen it is hard to believe that the distorted ones have any relationship to mitosis. It is not very satisfactory to demonstrate the figures in the living cells unless our critics are prepared to spend many hours with us searching through large numbers of fresh films, and patiently waiting until a series of figures are seen. We have found that onlookers are seldom prepared to do this.

With regard to the fixed films, however, there are hopes of ultimately obtaining a set of specimens of the induced division figures in all their phases. I do not assert that we shall be able to do this ourselves, because we hardly feel justified in devoting all our time, perhaps for months to come, in trying to get records of facts about which we are absolutely certain, and of which we now have confirmation by other procedures; but it is to be hoped that others will gradually try the jelly method and the fixation of the films, when a series of typical figures may be obtained for permanent record. The probabilities are that a very large number of films will have to be made, for in each jelly film from which the fixed specimens are prepared, the majority of cells may be distorted. There may, of course, be one or two typical figures, although even then the chances are that they may not be absolutely in a definite well-known phase of mitosis, and even if they are in one of the stages, the attitude (over which there is no control) which they present to the observer may not be suitable. Supposing there is one such cell, however, it may not ultimately

be fixed to the cover-glass, for during the process of fixation it may become achromatic and disappear, or it may, as many of the cells do, adhere to the jelly and not to the cover-glass on the removal of the latter. Again, it may not be in the zone of fixation at all, because, as already shown, the fixing osmic acid only penetrates a certain distance under the cover-glass. Furthermore, supposing that a film did contain a typical figure, the latter can easily escape observation. One can never be certain of examining every field, especially as high powers have to be used, and it is very easy to lose a cell again in spite of the fact that one's microscope is fitted with accurate verniers. There is no question that, if we are going to obtain records of lymphocytes with division figures induced in them resembling all the well-known phases of mitosis, we shall have to be blessed with extraordinary good fortune, unless many thousands of films are made and critically examined. I may mention that it occupied a whole year's work for two of us to obtain the photomicrographs of dividing cells which we have published, and none of them are either typical or satisfactory.

In the fixed specimens which we have already obtained, however, there are some good figures, which, although they are not actually in the conventional phases, are sufficiently close to one or other well-known stage that one can, I think, after having seen the living cells in all their stages and attitudes, as I have done, show their relationship to the phases and point

out the sequence of events. I will deal first with the four figures painted from the fixed films and reproduced in colours in our last publication. They are the most typical permanent specimens which we have obtained up to the present.

Before comparing the figures, I wish to reiterate that the induced division figures of lymphocytes are exactly the same as mitotic figures except that the chromosomes are formed, not from the chromatin " ids " within the nucleus, but from the chromatin granules, which, in these cells, are immediately outside the so-called nucleus and in the cytoplasm.

We may begin by comparing the best of our recorded figures, that of a polar aspect of mitosis in a fixed film (illustration No. III in *Further Researches, etc.*, Vol. I), with diagrams of similar

From Gray's *Anatomy*.

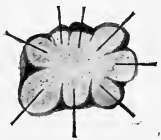
From a diagram of chromosomes in Ascaris egg. Wilson's *Cell in Development*.

From our *Further Researches*, Vol. I. September 1911, p. 38, Fig. III.[1]

[1] Fig. 5 in *Further Researches*, Vol. I, is a painting of another cell in a fixed film in almost precisely the same stage as this one.

aspects in Gray's *Anatomy*, and in *The Cell in Development and Inheritance*, by E. B. Wilson.

At first sight there appears to be no similarity between Wilson's or Gray's diagrams and our own illustration of the mitotic figure in the fixed lymphocyte. Both of the former are diagrams of cells in the metaphase, polar view, and both of the cells are in a slightly later stage of mitosis than the lymphocyte, which is in a transitional stage between the late prophase and early metaphase. As I have seen exactly how the chromosomes are formed in the lymphocyte, it is easy diagrammatically to place our illustration in a slightly later stage, namely, in the metaphase. Because the illustration is that of an early figure, the internal ends of the loops or festoons appear to meet at the centre of the cell, which in reality is the pole of it, or position of the centrosome. The continuation of the loops is due to the fact that not quite all the granules (ids) have yet collected at the equatorial plate ultimately to form the chromosomes, and some are still on their way, extending down in the grooves of the "spindle," which, of course, is somewhat flattened out owing to the fixation. Hence, when we now draw the cell in a later stage, these meeting lines must not be complete. Each loop or festoon divides transversely at its centre at the place indicated by the black lines in this diagram:



We may now compare once again the diagrams of Gray and Wilson with our figure in which the loops are divided to form chromosomes, thus depicting the cell in its early metaphase. As

in Gray's and Wilson's diagrams, the chromosomes are V-shaped, attached at their apices to the spindle, which, of course, is unstained.[1]

Thus, by converting the illustration of the fixed lymphocyte diagrammatically into a slightly later stage of mitosis, one can show how the chromosomes do to some small extent resemble those in the well-known diagrams, and it may be useful to reprint here two of the photomicrographs of living lymphocytes in the induced metaphases of mitosis (polar aspect) which we published in our first book, *Induced Cell-Reproduction and Cancer.*

In both of these photomicrographs, which of course are not nearly so demonstrative as the fixed films, one can, perhaps, observe a slight semblance to the more typical figures represented above. The photographs are those of cells which were actually in the metaphase, although owing to pressure, especially in Fig. 64,

[1] If the same procedure is adopted with Fig V. in *Further Researches*, Vol. I, a very similar result will be obtained.

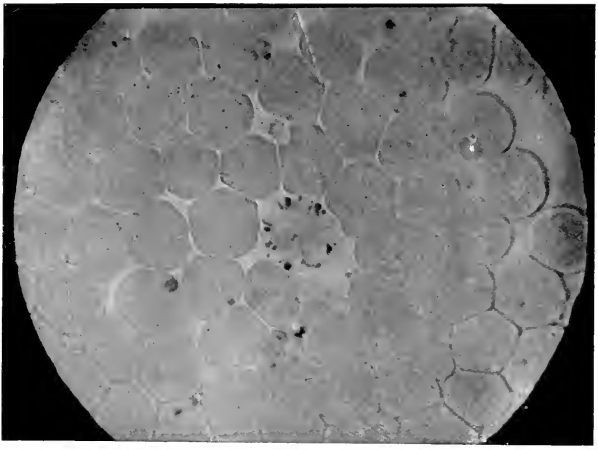


FIG. 63.

Polar aspect. The chromosomes were V-shaped with their apices inwards to be attached to the nucleus-spindle, which can dimly be made out.

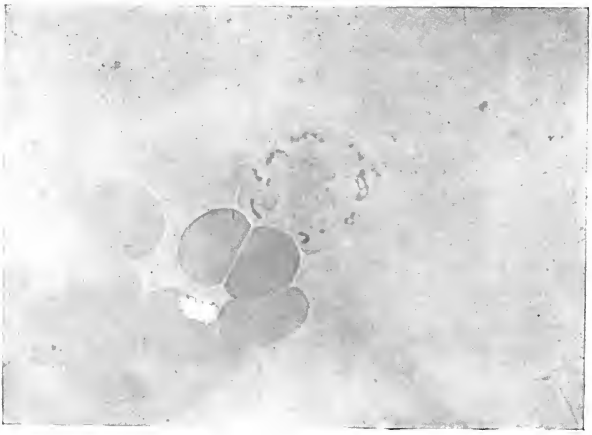


FIG. 64.

Polar aspect of mitosis in a large lymphocyte from a cancer patient. The chromosomes are dividing.

the chromosomes are rather distorted and have become separated.

With regard to the profile aspects of the division figures, Figs. II and IV in *Further*

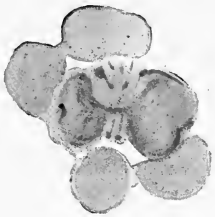
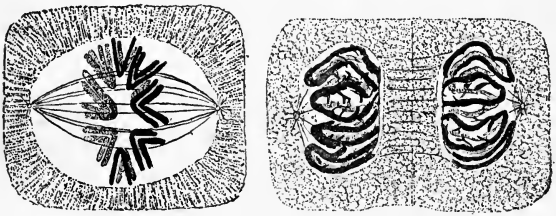


FIG. II.—*Further Researches*, Vol. I.

*Researches* depict cells which are in a transitional stage between the metaphase and telophase. As a matter of fact the cells are in the telophase, but they both show a few chromosomes still remaining at the equatorial plate (metaphase)



Metaphase. Telophase.

From Gray's *Anatomy*.

which have not yet dispersed into the daughter cells.

In reality, therefore, this figure is an " intermixture " of two phases, examples of which are depicted in Gray's *Anatomy*.

Or we may compare it with Wilson's diagrams:

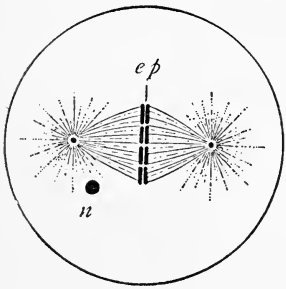



FIG. 26G.
Metaphase.

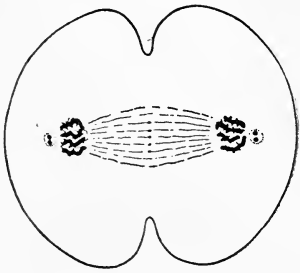


FIG. 26I.
Telophase.

From *The Cell in Development and Inheritance.*

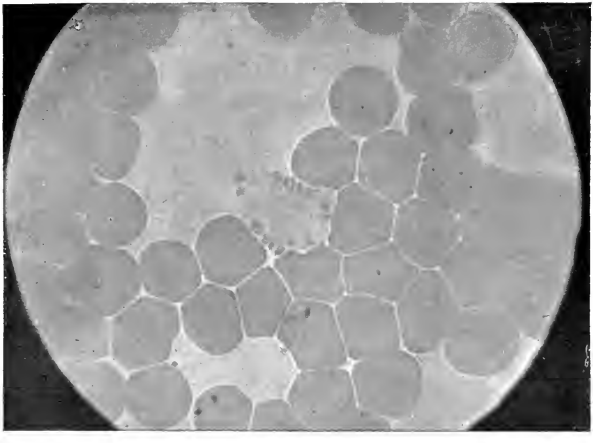
We can alter our figure diagrammatically, in a way similar to that adopted with the polar aspect figure, for, by deleting some of the ids on the one hand and the chromosomes on the other, we can show the same figure in the metaphase (or early anaphase) and in the telophase.

Photograph No. 67 in *Induced Cell-Reproduction and Cancer* is one of a living lymphocyte in the metaphase (or early anaphase), but owing to pressure, the cell has become rather flattened out. Photograph No. 70 is of a lymphocyte in the late telophase.

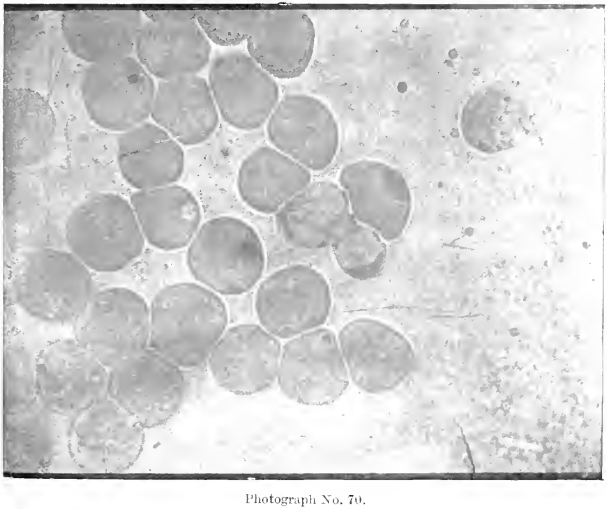
Metaphase (or early anaphase).

Telophase.

Our profile figures are all, of course, in a much later stage of mitosis than the polar-view ones



Photograph No. 67 in *Induced Cell-Reproduction and Cancer*



Photograph No. 70.

which we discussed first. For this reason, the so-called nucleus in the profile figures appears to be stained. In the polar view figures, practically all the chromatin granules have collected at the equatorial plate, because the cells are just entering the metaphase. As already described, a few ids still remain in lines radiating from the pole to the apices of the chromosomes, or rather of the loops which will ultimately form the chromosomes. Hence the nucleus or spindle is practically colourless. In the profile figures, however, the cells are leaving the anaphase and entering the telophase; a few of the ids still remain as chromosomes, but the majority of the chromosomes have broken up again into ids, which have become distributed over the surface of the daughter "nuclei." During the process of fixation, these chromatin granules have become flattened out and crushed into the daughter nuclei, giving the latter the appearance of being composed of deeply and more or less homogeneously staining chromatin. If a fixed lymphocyte is stained in its resting stage, the so-called nucleus appears to be a deeply stained chromatin mass, but if a living resting lymphocyte is stained by the *in-vitro* method, as already described in former publications, the so-called nucleus appears as a transparent membrane with no chromatin inside it, but studded all over its outside by minute chromatin granules.

It is unfortunate that it is so difficult to obtain records of the cells with the induced division figures in them, because otherwise I feel sure that many persons would try the new

methods, and see for themselves how the cells divide, rather than rely too implicitly on the conventional diagrams of cytology. We have done our best to obtain what records we can, but it is tedious work which only delays progress. One must admit that our records are poor, and it is not surprising that people ask for something better. Unless some remarkable new method of recording the specimens is invented, I think that the quickest way to overcome the difficulty is for those who are interested to employ the jelly method for themselves. Hitherto I have purposely refrained from trying to establish the induced figures by means of diagrams, because I considered that it was useless to expect people to believe in mere drawings when the subject of the induced division of human cells is so new. For this reason we resorted to every means in our power to obtain actual records of the cells themselves; but it is now two and a half years since the divisions were first induced, and although every effort has been made to obtain as many records as possible, it must be admitted that the results are not so satisfactory as one could wish. It is possible that if we went on for another two and a half years, while the chances are that we should get slightly better records, we might not meet with more success, and our specimens and photographs remain as unconvincing as before. Therefore, I now propose to describe with the aid of diagrams the division of human lymphocytes induced by auxetics as I have seen them, stained *in vitro*, but it should be remem-

bered that they are only diagrams, drawn from memory, and that we have no fixed specimens like them all.

In order to demonstrate the relative position of the structures, in all these diagrams the so-called nucleus has been more clearly outlined than is really the case.

Fig. 1 is a diagram of a resting lymphocyte. The so-called nucleus studded all over its outer surface by granules may be noted. These ids, judging by the way they stain with aniline dyes, are composed of chromatin. They are in the cytoplasm, and are frequently seen to be extruded into pseudopodia. Within the nucleus there is the ring-shaped nucleolus.

Fig. 2 shows a cell in the earliest prophase. It is slightly elongated, and the nucleolus has divided into two parts which are diverging towards the poles. The ids are comparatively deficient at the poles owing to the fact that they are beginning to collect in the neighbourhood of the equatorial plate.

In Fig. 3 the same condition is more marked, and the so-called nucleus is spindle-shaped. The nucleoli have adopted the positions of centrosomes. A few ids can still be seen extending downwards in lines from the poles to the equatorial plate, where the majority of them have collected.

Fig. 3a is the polar aspect of Fig. 3. The radiating lines of ids can be seen resembling those in Fig. III in *Further Researches*.

Fig. 4 shows a slightly later stage. The ids have become more consolidated both at the

equatorial plate and in the lines where they extended down to the poles; lines which are now no longer complete.

Fig. 4a shows how, when this stage is looked at from the pole, the ids have become arranged in indefinite festoons semilunar-shaped, eight in number.[1]

In Fig. 5 the ids have collected to form what appears to be a solid belt of chromatin at the equatorial plate.

Fig. 5a is the polar view showing that, owing to the festooning, the belt has a rosette arrangement.

Fig. 6 is the first step in the metaphase. Each festoon has divided transversely at its centre, but for the present each half thus produced remains united at its end with the end of half of its fellow festoon.

Fig. 6a shows this more clearly, and it may be compared with our diagrammatic alteration of Fig. III in *Further Researches* (*loc. cit.* p. 48). This transverse splitting of the festoons leaves the figure with eight V-shaped chromosomes, having their apices inwards attached to the nucleus-spindle.

Fig. 7 is the first one of the anaphase. The V-shaped chromosomes have divided at their apices.

Fig. 7a, the polar view, now depicts the cell with the chromosomes divided into sixteen portions.

[1] Throughout these diagrams the number of festoons, and therefore of chromosomes, is eight, which are commonly seen. In the induced figures, however, sixteen and thirty-two chromosomes may also be seen.

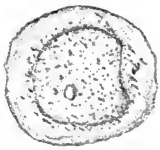


FIG. 1.

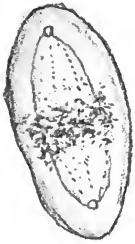


FIG. 3.

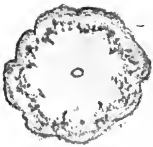


FIG. 4a.

FIG. 6a.

FIG. 6.

FIG. 2.

FIG. 5a.

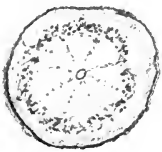


FIG. 3a.

FIG. 5.

FIG. 4.

Fig. 8 shows each portion gradually travelling towards a pole—eight to one pole, and eight to the other. (As the diagram is of a longitudinal section, only half this number appear in it.) While they travel, the rod-shaped bodies break up again into ids, thus reverting to their resting condition.

Fig. 9 is a later stage where the ids are diffusing over the surface of the daughter "nuclei," which are formed from the spindle. The latter has become constricted at its centre.

Fig. 10 is a diagram of the telophase. The daughter cells are formed and about to separate. The centrosomes have become invaginated within the "nuclei."

In Fig. 11 complete division has occurred. The chromosomes have entirely reverted into ids, which once more cover the surface of the so-called nucleus. The centrosome again appears as the nucleolus.

Such is the manner in which lymphocytes divide. The figures are similar to mitotic figures except that the chromosomes are formed from the granules which are immediately outside the so-called nucleus. This point at first sight appears to be very revolutionary, but there is no doubt that the nature of the granules differs sometimes in different classes of cells. For instance, the granules of liver cells are quite different and stain differently from those of lymphocytes and leucocytes. Yet these granules, in many classes of cell, have all been heaped together under the common name

of "Altmann's granules," and some people expect them all to have the same function. We have no proof that these structures in all classes of cell are derived from the same source, any more than we can assert that the structure known as the nucleus in one class of cell has necessarily the same function as that which is known as the nucleus in another cell until we have witnessed the life history of both classes. Take the case of the so-called polymorphonuclear leucocyte. Wharton Jones in 1846 first stated that these cells were "nucleated." He suggested that the curious-shaped body within these cells was the same thing as the nucleus in other cells except that it was polylobed. He had no grounds whatever for his statement, which really was in the nature of a speculation. But it was jumped at and accepted, and, although the cells had never been seen to divide, they were given the name of "polymorphonuclear leucocytes." Now that we can make these cells divide, and dispute the accepted idea of their so-called nucleus, we are attacked on all sides. I presume that the word nucleus originally meant a body inside another; but this is no criterion that all nuclei should have the same function.

Intranuclear centrosomes have been seen in other cells as in lymphocytes, and I have been informed that some of the spindle fibres in some cells appear to be formed partly out of the nuclear wall. Only recently, in a paper reported in *Nature*,[1] the author points out how during

[1] See *Nature*, November 9, 1911, p. 59. As far as can be ascertained, this paper has not yet been published in full.

FIG. 7.

FIG. 8.

FIG. 7a.

FIG. 9.

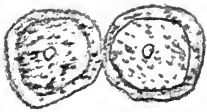


FIG. 11.

FIG. 10.

mitosis in certain cells, the nuclear wall shrinks and envelops the chromosomes. It is possible that the appearance of the spindle-*fibres* may partly be due to some *post-mortem* effect. I have never seen them in a living cell, although fixed films show some lines indefinitely resembling them.

Critics have suggested that lymphocytes are sometimes found in the act of division in sections of lymphatic glands, and we have been asked why our figures do not resemble them. But if these sections are not stained too deeply, and some resting lymphocytes in them very carefully compared with the mitotic ones, it may be seen that the chromosomes are formed out of the granules. It is as well to ask oneself what has happened to the granules in the dividing cells in the sections, for they no longer appear to be visible. In reality they have formed the chromosomes.

In conclusion, I should like to take the opportunity of mentioning experiments which show that the division of white blood-cells does not appear to depend on alterations of surface tension. We have been asked about this point on several occasions. Jellies have been prepared which contain different strengths of saponin, sodium glycocholate, sodium taurocholate, and oleic acid, and their action tested on the living cells in precisely the same way in which we test auxetics. With none of them has any action been visible, except that if they are present in strong solution, they burst the cells. They do not excite amœboid movements (kinetic action), and they certainly do not induce division figures.

## IV

# EXPERIMENTAL CELL-DIVISION INDUCED IN THE EGGS OF *ASCARIS MEGALOCEPHALA* BY SUBSTANCES SET FREE BY CELL-DEATH: ANOTHER EXAMPLE OF THE ACTION OF AUXETICS ON INDIVIDUAL CELLS

By H. C. Ross and J. W. Cropper

As described in the former volumes, cell-division has now been induced in lymphocytes, polymorphonuclear leucocytes, and some epithelial cells *in vitro* by the agency of auxetics. The hypothesis based on this work is that the chemical substances set free by cytolysis are the direct cause of normal cell-reproduction; an hypothesis which has been borne out by the *in-vivo* experiments already published, and especially the experiments on animals which were enumerated in the first chapter in this volume. Unfortunately, as described in the preceding chapter, the method employed in the *in-vitro* experiments, by which individual cell-division was first induced in lymphocytes, disclosed a revolutionary fact about the division figures of these cells. This led to scepticism,

as already mentioned, and some pathologists said that since the division figures in lymphocytes did not accurately resemble the well-known pictures of mitosis, they could not believe that it really was cell-division which was being induced by the auxetics, and that they wanted more proof. In spite of the fact that Dr. Fantham has also succeeded in inducing cell-division in *Entamœba coli* by means of auxetics, which is very strong confirmatory evidence, our interpretations are still disputed by many persons. Dr. Fantham has published the fact that not only can individual amœbæ be made to divide with the jelly method by the action of auxetics, but also that these parasites can be cultivated through several generations on a medium which contains, in addition to salts, only an auxetic substance such as tyrosin. Hitherto, it was necessary to transplant the amœbæ on to a fresh nutrient medium after each generation of life-cycle was complete, but now, by means of auxetics, generation after generation can be cultivated without transplantation.[1] Auxetics seem to cause the whole cycle of reproduction in *Entamœba coli* in the same way in which they appear to be responsible for the cycle of cell-division in lymphocytes, polymorphonuclear leucocytes, and certain epithelial cells of human beings. Still, as more proof was asked for, we have experimented with several forms of

[1] The papers by E. H. Ross towards the end of this volume give further evidence regarding the action of auxetics on protozoa.

ova in order to try to provide it. The eggs of *Ascaris megalocephala* have proved to be suitable for this kind of experiment.

These ova can be obtained as follows: Any abattoir in which horses are slaughtered can supply the worms, which will live for a day or two *in vitro*. It is advisable, however, to use the parasites as soon as possible after they have been procured. A worm should be slit up with a pair of scissors, and the long double uterus exposed. The latter is easily removed and divided. By means of a little pressure, the ova, surrounded by a white viscous substance, can be squeezed out from one of the cut ends of the uterus, and they can afterwards be teased out in a little water in a watch-glass. With a platinum loop samples can be removed on to a slide or cover-glass for examination by the microscope.

The eggs seem to consist of a thick wall surrounded by a coating of mucus. Inside the wall there is a clear layer, and immediately within this again there appears to be a fibrous membrane. Within this layer once again is an indefinite mass of granules, and sometimes embedded in them the round or oval nucleus can be made out. The examination of thousands of these ova shows that they practically always present this appearance. Only on one or two occasions have we seen cell-division going on in the eggs when they were removed.

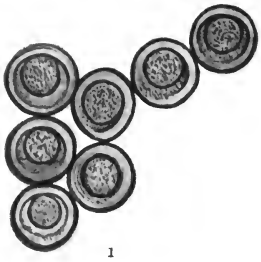
In order to induce cell-division in them, the best procedure has been to place the ova

directly into a few drops of the solution of auxetic in a watch-glass. The watch-glass is kept in a petri-dish, which, in its turn, contains a few drops of water to prevent evaporation of the auxetic solution; and the petri-dish is kept for about twelve hours in the 37° C. incubator. The auxetics with which we have succeeded in inducing cell-division in these ova have been extracts of a tissue such as suprarenal gland (100 grammes of gland in 100 c.c. of water, extracted for twenty-four hours and then filtered) and a solution of creatine (4 per cent). At the end of the twelve hours many of the ova exhibit cell-division.

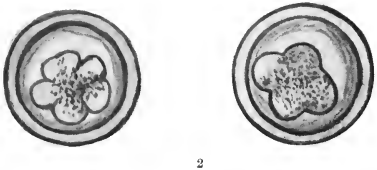
The former researches have shown that such substances as choline and cadaverine greatly augment the action of auxetics. If the extract is allowed to become putrid, or if 1 per cent of cadaverine is added to the creatine solution, every ovum will show advanced cell-division in about eight hours; and in our experiments, as far as could be seen, several generations, certainly more than eight, of the daughter cells were produced in the eggs.

Care was taken in each experiment to make accurate controls, which revealed the fact that the exact part of the uterus from which the eggs are taken is of the utmost importance. If they are removed from near the genital opening it will be found that cell-division will occur at 37° C. in all the ova whether they are subjected to auxetic or not. On the other hand, if the ova are removed from the other end, they appear to be undeveloped, for their fibrous layer seems to

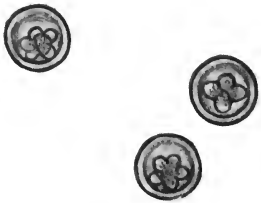
be wanting, and so far by no agent have we been able to induce cell-division in these premature eggs. It is necessary to remove the ova from a point between two and a half inches and three inches from the opening. If samples are taken from this point—which can only be accurately determined by experiment—eggs will be found which rarely (perhaps 2 or 3 out of 500) divide in water or saline, but which will nearly all divide in auxetic solution. The contrast between the eggs in which cell-division was induced and those in the control watch-glasses was so striking as to leave no room for doubt that cell-division had actually taken place, and that it was induced by the action of the auxetics (see illustration). Moreover this contrast is sometimes evident in single specimens. When a small mass of ova—which have not been very carefully separated by teasing out—has been placed in auxetic solution (such as putrid extract) which contains a brown colouring matter, some of the eggs are in a position to absorb more auxetic and colouring matter than the others. This is probably due to their being nearer to the surface of the mass, and it is demonstrated by their deeper coloration. When many such specimens have been looked at, it becomes apparent at once that those which are more deeply coloured have, in a great majority of instances, undergone further development than those individual ova which have remained of light colour, and which have been in contact with less auxetic. For instance, in three specimens the figures were as follows:



1



2



3

THE EGGS OF *ASCARIS MEGALOCEPHALA*.

1, Control specimen: these eggs had been kept in salt solution for 12 hours at 37° Cent. 2 and 3, Auxetic specimen: these eggs had been kept in extract of a tissue (auxetic solution) for 12 hours at 37° Cent. 2, higher power; 3, lower power.

| Specimen No. | Colourless Cells (less auxetic) | | Deeply Coloured Cells (more auxetic) | |
|---|---|---|---|---|
| | Dividing through not more than One Generation | Dividing through Two Generations or more | Dividing through Two Generations or more | Dividing through not more than One Generation |
| 1 | 275 | 5 | 121 | 18 |
| 2 | 181 | 1 | 57 | 21 |
| 3 | 33 | 2 | 35 | 27 |

We submit that the majorities are sufficiently great to exclude fallacy owing to the error of random sampling.

The above has been found to be the simplest technique. Attempts to induce the cell-division with jellies containing the auxetic have so far not been successful, probably because the jellies evaporate, and only one side of the ova is in contact with them, thus preventing complete diffusion of the auxetic—the other side of the eggs being in contact with the cover-glass. Azur stain, however, will also induce cell-division up to one generation in the ova. It may be remembered that, as we have already pointed out in former papers, this dye will also induce cell-division in lymphocytes. It probably acts by producing the gradual death of protoplasm immediately within the walls of the ova, setting free the auxetics which cause cell-division—a theory which has already been suggested by us.

These experiments were undertaken in order to prove that cell-division is caused by the chemicals set free by cell-death in accordance with the main argument of our hypothesis; we do not wish to insinuate that this is the first occasion in which cell-division has been

induced in ova; for, as is well known, Jacques Loeb, and after him several other persons, induced development in eggs of sea-urchins several years ago. Loeb used sugar, butyric acid, and other artificial substances, which in all probability acted in a way similar to azur stain. In fact, Loeb himself has recently pointed out that "all agencies which cause a definite type of cell-destruction—the so-called cytolysis —cause also the egg to develop, as long as their action is limited to the surface layer of the cell." [1] And he suggests that this explains the action of butyric acid. We go a step further and suggest that it is the products of cytolysis which cause the development.

[1] "The Mechanistic Conception of Life," by J. Loeb. *The Popular Science Monthly*, January 1912.

# THE GASWORKS PITCH INDUSTRIES AND CANCER

By H. C. Ross and J. W. Cropper

In our last publication (September 1911) it was pointed out that auxetics and kinetics, the combination of which, according to our working hypothesis, is the cause of cancer, had been found in gasworks tar and pitch. They had also been found in soot, but not in blast-furnace tar and pitch. Warts and ulcers which sometimes become epitheliomatous occur among the workmen in those patent fuel (briquette) manufactories which use pitch made from gasworks tar, but not in the works which employ blast-furnace tar pitch. It was mentioned that an experiment had been made for us by one of the Patent Fuel Companies in order to find out whether the auxetics and kinetics could be eliminated from the pitch by washing (for they are soluble in water) without injuring the pitch. The pitch was ground up and mixed with water until no more auxetics or kinetics could be detected in it, and it was then dried and used in the ordinary way for the manufacture of briquettes. Unfortunately the washing ruined the pitch;

its " binding " quality had greatly deteriorated, and the briquettes made from it had very poor " steaming power " when used in a locomotive. Pitch is the residual mass left in the retorts after distillation of tar, and therefore, since nothing practical could be done with the pitch, we suggested that experiments might be made with the tar to see whether, having removed the dangerous principles from it, the pitch made by its distillation would still be useful commercially.

It was also mentioned that an Inquiry was being held by the Home Office to consider objections raised by the Patent Fuel Companies against some regulations which the Factories Department of the Home Office proposed to make with the object of preventing the incidence of the warts and cancer amongst the workmen. The regulations consisted of the establishment of bathing accommodation in the works and the providing of overalls for the men to protect them from the pitch dust. In our publication we suggested that, if possible, before the regulations were imposed, experiments should be undertaken to find out whether the auxetics and kinetics could be eliminated from the pitch or tar, because, if this could be accomplished practically, and it was subsequently found that the pitch so treated was harmless, not only would the regulations be unnecessary, but also strong circumstantial evidence would thereby be provided that the combination of auxetics and kinetics is the cause of malignant cell-proliferation in accord-

ance with our working hypothesis. On the other hand, if the regulations were immediately imposed, they would render fallacious any experiments made to eliminate the dangerous principles, because, supposing good results were obtained, it would be impossible to state whether they were due to the treating of the tar and pitch or to the regulations. The Report of the Inquiry has now been published.[1]

In this Report, after describing the extent of the patent fuel industry, and the danger of corneal ulceration occasioned by it, Mr. Lush continues as follows :

" All fuel workers are exposed in some degree to the action of pitch dust on the skin, the pitchworkers from the pitch, the factory men from the dust in the factories, and those who deal with the briquettes from the pitch therein contained. The effects of this dust are of three kinds, but these seem to represent three stages of the same process of irritation by pitch.

" In the first place there is a burning sensation in the face and extreme sensitiveness to the action of the sun or keen winds. This stage is painful, but not dangerous to health, and is almost universal, only a small percentage of fuel workers being free from it.

" After a considerable period, usually several years, of working there is a tendency for a peculiar kind of warts known as pitch warts

[1] *Report to His Majesty's Secretary of State for the Home Department on the Draft Regulations proposed to be made for the Manufacture of Patent Fuel (Briquettes) with addition of Pitch.* By Alfred Herbert Lush, Barrister-at-law. (1911.

to form on the face and neck and various portions of the body. Out of 246 fuel workers examined by Dr. T. M. Legge, of the Home Office, 51 were found to be then affected by these warts, and 28 showed signs of having formerly been affected. Presumably these men included some who were not much exposed to the dust, and others who had not been working long enough for the warts to develop, so that the proportion of men amongst the fully exposed class who sooner or later become affected may be considerably larger than these figures indicate.

" Usually these pitch warts are not malignant in character, but when they occur in particular parts of the body—for instance, the lips or the genital organs, and more especially the scrotum—there is a distinct liability to a third stage, namely, the form of cancer called epithelioma. Scrotal epithelioma is of interest and importance for the purposes of this Inquiry, because it is a rare disease amongst the general population, but by no means rare amongst chimney-sweeps, who are exposed to soot in the same way as fuel workers are to pitch dust.

### " Suggested Causes of the Disease

" The origin of the trouble is believed to be the blocking of the sebaceous glands and hair follicles by minute plugs of pitch, but how exactly this operates to produce cancer is not clear. Drs. H. C. Ross and J. W. Cropper, in an article in *The British Medical Journal* of April 15, 1911, suggest that there are chemical

agents in the pitch which directly induce the abnormal cell-division characteristic of the disease. Sir H. T. Butlin, an unquestioned authority, appeared to doubt this theory, and regarded the irritation set up by the pitch as the cause of the cancer.[1] I am in no way qualified to judge of such a matter, but I may point out that, so far as the evidence goes, the cases of epithelioma in fuel workers do not arise immediately from the pitch dust, but only as a secondary result of pitch warts so situated as to be exposed to irritation. It is agreed that the disease is local in its origin—that is to say, it is due to dust which has settled on the actual part affected."

Mr. Lush then goes on to state the extent of disease amongst the workmen, and comes to the following conclusion :

" Nevertheless I find it impossible to regard the danger as otherwise than a serious one. It cannot reasonably be contended that warts which may develop into epithelioma, unless they are removed by the surgeon's knife, or subjected to some process of burning off, are not dangerous to health, and I am satisfied by Dr. Legge's evidence that such warts are widely prevalent at least amongst the pitch workers and the men who manufacture the briquettes. The case for regulations so far as relates to

[1] It is difficult to reconcile this opinion of the late Sir Henry Butlin with his theory that cancer cells are parasites " coming from within," and that the disease is essentially parasitic in origin in the way described in his Hunterian Lectures entitled *Unicellula cancri, the Parasite of Cancer* (Nov. 1911).—H. C. R. and J. W. C.

those men has therefore to my mind been established."

The Report also describes the proposed regulations in detail—namely, the prevention of dust in the factories by encasing the machinery, and the protection of the men's bodies from dust by means of overalls, and by the establishment of bathing and washing accommodation at the works. The attitude of the workmen towards the regulations, and the objections raised by the employers against them, are subsequently discussed; and it was ultimately proposed that the institution of the regulations should be postponed for experiments to be made.

"This proposal was that before the regulations are finally settled time should be allowed for experiments to determine the various questions now unsettled, and on certain conditions I am prepared to recommend that it should be adopted. The considerations which mainly influence me are the uncertainty as to what is really required to remove pitch from the skin, the difficulty as to the pain experienced after washing, and the consequent objection of a large number of men to submit to the regulations until that difficulty can be removed. If in addition to experiments in these directions the difficulties connected with the subjects of overalls and goggles could be further investigated, the results might be of great service.

"The conditions I think necessary are these. Experimental baths on a moderate scale to be installed as soon as practicable at one or more

of the factories, if possible one at Swansea and one at Cardiff. Volunteers to be sought amongst the men to give the baths a fair and complete trial, each man to take a bath daily for at least a week, and until further experience be gained only one man in a shift to be allotted to a bath. The shippers, who seem to be the natural leaders of the men, to be allowed to volunteer. Suitable arrangements for clean clothing to be made. Reasonable washing accommodation, even if of a temporary and make-shift character, to be forthwith provided for the men likely to need it. Lastly, all practical measures to be taken in hand forthwith to reduce the excessive dust found in some of the factories. The Factory Inspectors must be allowed to watch the experiments when they desire it, and the occupiers must undertake to take proper notes of the results of the experiments, and to furnish these notes to the Home Office in such form as may be required by the Chief Inspector of Factories."

In the penultimate clause of the Report Mr. Lush makes the following statement, which is in accordance with the views originally suggested by us :

" If in the meantime the mischievous agent in the pitch should chance to be detected, and a means of eliminating it discovered which is commercially practicable, that would afford a more satisfactory solution of the difficulty than can possibly be attained by these regulations."

In the meantime we had been continuing

our experiments. Watery extracts of gasworks tar and pitch were directly inoculated into the subcutaneous tissues of experimental animals, with the result that cell-proliferation and tumours were always produced at the sites of inoculation. Microscopical sections showed the tumours to be of the nature of granulomata and fibromata. On the other hand, extracts of blast-furnace tar and pitch do not produce tumours at all comparable in size or time occupied in their appearance to those produced by the gasworks tar, in fact only slight proliferation of healing takes place at the site of the injury. These experiments in our opinion are very strong confirmatory evidence that the mischievous agents in the gasworks tar are the auxetics and kinetics; and they almost conclusively dispose of the idea that the diseases caused by the pitch are set up by the "irritation" of the pitch dust, for no dust of any sort was present in our experiments.

Since the institution of the regulations has been postponed and there is now time for further experiment, we again communicated with Sir Arthur Whitelegge, the Chief Inspector of Factories at the Home Office. It was explained to him that it was useless to experiment any more with the pitch because, as already mentioned, mixing it with a liquid ruins its commercial value. We suggested that some experiments might now be carried out on the tar with a view to ridding it of the dangerous ingredients before it was distilled

to form pitch. It was pointed out to him that the experiments ought to be done on a larger scale than we could manage in the laboratory owing to lack of plant and material. Sir Arthur Whitelegge very kindly placed us in communication with the firm of Messrs. Bird & Son, tar-distillers at Cardiff. In the first instance we asked Messrs. Bird & Son whether they considered that it would be possible to wash tar prior to its being distilled, and we obtained the following information.

It seems that tar is the residue after fractional distillation of coal, and pitch is the residue after fractional distillation of tar; and about four-fifths of all the pitch made is sold to the briquette works. Briquettes are made by mixing and compressing small coals with powdered pitch. Practically every substance produced by the fractional distillation of tar, viz. the paraffins, naphthas, creosotes, etc., are made use of commercially. The tar-distillers purchase the tar from different gasworks all over the country according to the fluctuation of market prices. The quality of the tar varies very considerably, its chief impurity being carbon (soot). It appears that tar is merely considered as a by-product at the gasworks; their chief commodity being, of course, the coal-gas which is supplied for town lighting and heating. So long as the gasworks obtain their proper quality and quantity of gas, they do not pay much attention to the quality of the tar, which is merely sold in bulk to the distillers. The tar may contain

as much as 50 per cent of its impurity, sometimes it is so thick with soot that it will only with difficulty run out of the vents in the railway trucks; and the percentage of impurity is due to many factors—the quality of the coal, the temperature and manner of distillation, and the kind of retort employed.

It is the presence of this impurity which has frustrated our progress in the attempt to rid the tar of its dangerous principles, for, as the kinetics and auxetics are soluble in water, we suggested that the tar might be washed with water prior to its distillation. But Messrs. Bird & Son showed that the presence of water in tar is the one thing dreaded by tar-distillers. Every effort is made by them to keep tar free from water, because if it is present when distillation takes place an emulsion is formed—between the soot and the water—which froths up out of the retorts. This involves prolonged and very gradual distillation to drive off the water slowly before actual distillation of tar can occur, requiring the use of more fuel and labour with their attendant increase of expenditure. Moreover, one of the valuable products of the distillation of tar is the ammoniacal liquor. If the tar is mixed with water this liquor is so diluted as to entail great loss. Therefore mixing water or in fact any liquid with the tar prior to its distillation is out of the question.

The next suggestion was that the auxetics and kinetics might be precipitated from the

tar by means of alkali. As described in *Further Researches into Induced Cell-Reproduction and Cancer*, the auxetics and kinetics in tar are soluble in acids, but insoluble in alkalies. We therefore experimented with a small quantity of tar in the laboratory by adding to it some powdered caustic soda (sodium hydrate). When the mixture was boiled and a watery extract made from it, it was found that the auxetics and kinetics were not sufficiently precipitated to prevent the extract inducing cell-division in lymphocytes under the microscope; but when the mixture of tar with 5-per-cent caustic soda was roasted, as it would be during a process of distillation, the auxetics and kinetics were precipitated to such an extent as to render the tar inert to induce cell-division.

We therefore again approached Messrs. Bird & Son for their opinion regarding the practicability of mixing the powdered alkali with the tar before it is distilled. Messrs. Bird & Son stated that it might be tried in the first instance on a larger scale experimentally, although new plant would be required to ensure mixture of the alkali with the tar; but a far more important objection was pointed out by them—namely, that to undertake such a process as a routine would cost at least twice as much as the total profit which is ever made by the distillation of tar, an undertaking which, of course, under ordinary trade conditions would be prohibitive.

Supposing such a process had been practicable commercially, in order to prove on a large scale whether the auxetics and kinetics are

actually the cause of the warts and epithelioma, the following experiment would have to be made. It would be necessary for one firm of tar-distillers to agree to manufacture pitch made from tar which had been previously mixed with caustic soda. It would also be necessary for one of the patent fuel companies to agree only to use pitch obtained from this one tar-distilling firm. The incidence of inflamed skin, warts, and epithelioma having been carefully noted for some time prior to the experiment, a comparison would have to be made with it after the lapse of, say, three months, during which time only treated pitch was used in the works. The other patent fuel companies would act as controls, obtaining ordinary untreated pitch from any source except the one firm of tar-distillers which had agreed to make the pitch from treated tar. If it was found that the burning of the skin, warts, and epithelioma no longer occurred, or if their incidence was greatly diminished amongst the workmen employed by the patent fuel company which used the treated pitch, not only would a clue be given how to formulate legislation for the abolition of the dangers of the pitch industries, but also there would be practically conclusive evidence that auxetics and kinetics are instrumental in the cause of cancer.

Unfortunately it seems that the profits made by the tar-distilling and patent fuel industries are not very great, and any additional expenditure imposed on the companies would

be a serious matter, for it might mean the closing of some of the smaller patent fuel companies, which now barely pay their way, with the loss of employment to many workmen. Under these conditions one can hardly expect private enterprises to undertake experiments on a large scale, especially as the experiments can only mean pecuniary loss to the companies concerned. The greater loss would fall on the tar-distiller, whereas, even if the experiment proved successful, the gain would be in the health of the workmen engaged by the patent fuel companies only, and not in the workmen employed by the tar-distillers. Extra expense would also fall on the patent fuel company, however, for instead of being able to buy pitch from different distillers at market prices, it would only be able to purchase it from one firm at an increased price.

The evidence in our hands that these auxetics and kinetics are the cause of cell-proliferation and epithelioma is now very strong, especially in view of the recent experiments on animals (*loc. cit.* p. 34), and we think that there can be little doubt as to the fact that these same bodies found in the tar and pitch are the cause of the trouble in the industries concerned with their manufacture and working. Hence the question now arises whether we are justified in approaching the Government and asking it to consider the possibility of its defraying the expenditure connected with the experiment on a large scale described above. If the experiment were successful, it would show at once the

rational method of dealing with the incidence of disease caused not only in the briquette manufacture, but also in the tarworkers, gas-workers, coal miners, and chimney-sweeps; for we have been informed that the burns, warts, and even epithelioma also occur to some extent in these trades—in fact, it is believed that the proposed regulations, if they had been passed for the patent fuel companies, were going to be extended to the other industries. Moreover, we believe that the result would be the cessation of the somewhat extravagant speculations and theorising which are now taking place amongst some medical men and scientists as to the immediate causes of cell-proliferation and cancer, speculations which only add to the bewilderment of those engaged in experimental research, and which fruitlessly raise the hopes of those unfortunate individuals who suffer from the disease. We are confident that such an experiment, if successful, would lead all cancer research in one direction.

But although we are sanguine of success, the experiment might end in failure—in which case the money would have been absolutely wasted—and the state of affairs remain as it does to-day. It is doubtful whether a Government would consider expenditure under these conditions. At the same time we do not believe that such expenditure could be defrayed by a Research Fund, for it would hardly be fair to impose upon it the liability which such an experiment might involve. In our opinion, the interests of too many persons would be included

for such a responsibility to be incurred by individual philanthropists.

There is an alternative which was suggested by Messrs. Bird & Son. If tar did not contain its impurity, or even if the content of soot was reduced to less than 5 per cent, there is no reason whatever why it should not be washed with water at a very small expense prior to its being distilled, in which case it would appear that all difficulty would be removed. For some years past a new invention, a *vertical* retort for the distillation of coal, has been installed in two or three gasworks in the country. It is claimed that these vertical retorts produce tar which is nearly pure. If they were established all over the country, and if washing of the tar were enforced by legislation, the dangers of the industries would probably be removed. But this will take many decades in its accomplishment, because impure tar is nearly as valuable commercially as the pure quality, and no gasworks will install new plant until the old one is worn out. We hope to investigate this alternative further, for some experiments might be made with the purer tar, and in the meantime do not despair of finding some simple and, if possible, inexpensive method of treating the tar to rid it of its dangerous auxetics and kinetics.

## VI

# THE INHIBITORY EFFECT OF THE BLOOD-SERUM OF HEALTHY PERSONS ON THE ACTION OF AUXETICS IN INDUCING CELL-DIVISION

By J. W. Cropper

It has been shown[1] that certain human cells can be caused to divide on a microscope slide when they are made to absorb certain chemical agents from a jelly-film on which they have been placed. This excitation of reproduction was brought about in the first place by the use of aqueous extracts of various organs and tissues of the body, and subsequently the active principles were isolated from the extracts and found to consist of the products of the disintegration and autolysis of dead cells. The best known, and, from our point of view, the most important of these substances, are the purin bases and the amino-acids. Not only the natural bodies belonging to these groups, but, in addition, certain chemical substances having closely allied chemical structures (such as the amidines) have also been found capable of inducing division in individual living cells.[2]

[1] *Induced Cell-Reproduction and Cancer*, 1910.

[2] *Further Researches into Induced Cell-Reproduction and Cancer*, Vol. I, 1911.

As was already shown in a former publication (*Induced Cell-Reproduction and Cancer*, Chap. XVII), blood-serum has a power of restraining the action of these auxetic substances. The points which led to the investigation of blood-serum to see whether it has this inhibitory action will be found in the former publication, and will be mentioned again later, but the experiments made then showed conclusively that blood-serum does restrain the action of the substances which induce cell-division *in vitro*, although we have no opinion to offer as to what exactly this restraining body may be or how its action is brought about.

The object of the experiments now to be described was to investigate this inhibitory action further, especially with a view (1) to finding out the relative power of this action in the sera of different persons, and (2) to try to isolate the actual substance.

Although the technics for inducing cell-division on a miscroscope slide have been described on several occasions in our books and papers, it is necessary to repeat them here in order that the experiments can be clearly understood. A stock of neutral 3 per cent. agar jelly made with distilled water is prepared and sterilised. This is distributed in quantities of 5 c.c. into test-tubes, and to each of them is added 0·1 gramme of sodium citrate and 0·08 gramme of sodium chloride, and 0·008 gramme of citric acid. The mixture is known as "coefficient jelly," and it constitutes the jelly basis with which all the

experiments to be described were carried out. Each tube of 5 c.c. of coefficient jelly can be made up to a total of 10 c.c. by the addition of solutions of any chemicals the action of which it is desirable to test on the cells. The mixture is then boiled and poured while molten on to a microscope slide, where it will set solid at the room temperature. Living cells (*e.g.* white blood cells, parasites, cells of tissues, organs, etc.) suspended in a liquid medium can now be placed on a cover-slip which is inverted and allowed to fall gently on to the jelly film, when the cells will be found to be uniformly disposed and gently pressed into the jelly. By a correct adjustment of the alkalinity of the jelly, the amount of salts, stain, and chemical substances present, the diffusion of the substances from the jelly film into the cells can be controlled and the results noted. If auxetics are added to the jelly in correct amount, the cells will absorb them, and if the rate of diffusion is regulated to the proper degree, the cells will divide.[1]

As far as the following experiments are concerned, the cells employed in every case were human lymphocytes from the peripheral blood, because, as already mentioned in previous papers, they are less fragile than other cells and can be easily obtained. When the fresh blood is spread out between the cover-glass and jelly film, the lymphocytes can easily be recognised, even when no stain is present,

[1] The way of making this jelly will be found described in detail in *Induced Cell-Reproduction and Cancer*, Chap. VI.

from the fact that they are smaller than the polymorphonuclear cells, and do not contain the same large characteristic granules. The polymorphonuclear cells can also be made to divide in response to auxetics, but the technics are more difficult, and therefore these cells were neglected in the experiments to be described.

The fact which led H. C. Ross to suggest that normal serum contained some substance which restrained the action of auxetics in causing cell-division was that human leucocytes removed from the healthy peripheral circulation are never seen in the act of division. It is significant that normal leucocytes so removed always appear as spherical, not dividing cells, the lymphocytes never show mitotic figures, and yet it is known that auxetics do exist free in the circulation. The question therefore immediately arose as to why these auxetics do not cause cell-division amongst the leucocytes and lymphocytes, and make them exhibit stages of division on removal from the body. Hence it appeared possible that there might be some constituent of the blood-plasma which was capable of restraining the action of the auxetics which are present, or it might be some property of the plasma as a whole, due to its mechanical interference with the diffusion of the auxetics into the cells which are bathed in it. It was at this suggestion that the following *in-vitro* experiments were carried out to see if normal blood serum does exert any inhibitory effect on the divisions induced in lymphocytes by means of auxetics.

[1] *The variation in the restraining power of different persons' sera.*—An auxetic jelly containing suprarenal extract having been prepared which caused divisions in the majority of lymphocytes placed on it, fresh normal sheep's serum was added to it, and it was found that 2 c.c. of this serum completely stopped the divisions caused by 0·2 gramme of dried suprarenal extract. Another jelly contained 1 c.c. of creatine, and 1 c.c. of a 1 per cent. solution of choline (a powerful auxetic combination), and 2·5 c.c. of serum completely stopped the action of this also.

*Technique.*—About 5 c.c. of freshly-drawn blood was allowed to coagulate, the exuded serum decanted off and finally clarified by centrifuging. A piece of narrow glass tubing was drawn out to a point and graduated into tenths of a c.c. by calibration with mercury. The serum was measured in this previous to its addition to the auxetic jelly.

In the first place it was necessary to prepare an auxetic jelly on which at least 75 per cent of the lymphocytes exhibited signs of division, and for this purpose it was essential to adjust correctly the proportions of auxetic and alkali in the jelly. In these experiments the method of preparing the tube of auxetic jelly had to be slightly modified from that usually adopted, in order to permit of the addition of the large amount of serum, and because the latter cannot, of course, be added to boiling jelly or it will

[1] These experiments have already been described in detail in *Induced Cell-Reproduction and Cancer*, pp. 250–255.

coagulate. It was found convenient to have 4 c.c. of the coefficient jelly instead of the customary 5 c.c., and the auxetics, alkali solutions, etc., were added up to a total of 7 c.c. instead of 10 c.c.; 0·7 c.c. of this jelly was now taken and made up to 1 c.c. by the addition of 0·3 c.c. of distilled water and tested. If found satisfactory as an auxetic, serum in tenths of a c.c. at a time could now be added to the jelly as it cools, and the balance made up to 1 c.c. with distilled water.

Example :

*Auxetic Jelly.*

| | |
|---|---|
| Coefficient jelly . . . . . | 4·0 c.c. |
| Suprarenal extract 10 per cent . . . | 0·3 c.c. |
| Alkali (sod. bicarb. 5 per cent) . . . | 0·6 c.c. |
| Water . . . . . | 2·1 c.c. |
| | 7·0 c.c. |

*Experiment.*

| Control. | | Serum. | |
|---|---|---|---|
| Auxetic jelly . . | 0·7 c.c. | Auxetic jelly . | 0·7 c.c. |
| Water . . . | 0·3 c.c. | Water . . | 0·1 c.c. |
| | | Serum . . | 0·2 c.c. |
| | 1·0 c.c. | | 1·0 c.c. |

By this means the amount of serum employed in the experiments was varied, although the total amount of jelly employed in both control and test experiment was the same, *i.e.* 1 c.c., the balance of distilled water being varied to make up the total. The experiments were made in duplicate throughout with each strength of serum tested, and the original auxetic jelly was tested at the end of each experiment, because, as already stated, the

adjustment of the proportions of the constituents of the tube of auxetic jelly is a delicate matter, any deficiency or excess of auxetic or alkali destroying the power of the mixture to induce cell-division. The repeated boiling of the same tube of jelly causes concentration, so that care must be taken to prevent evaporation from the boiling test-tube. Hence the jelly was always re-tested after each series of experiments to determine if it was still effective as an auxetic. Each sample of serum was tested by adding first 0·1 c.c., then 0·2 c.c. and then 0·3 c.c. In a few cases loss of material necessitated a curtailment of these measures. The jelly was always allowed to cool to 45° C. before the serum was added, in order to prevent coagulation of the latter.

Using human serum, it was found that 2 c.c. stopped 0·2 gramme of suprarenal extract; 1 c.c. of serum stopped the action of 0·01 gramme of creatine and 0·01 gramme of choline (an augmentor); and 1 c.c. of serum stopped the action of 0·5 c.c. of a 2-per-cent solution of putrid "globin."

Atropine and other alkaloids excite amœboid movements in leucocytes, as already described; but it was found that serum does not stop the action of these substances. The action of choline, however, which also excites amœboid movements, is completely stopped by serum. Some aniline dyes, such as azur, induced cell-division, but serum does not stop their action.

Thus it appears that normal serum does actually possess the power of restraining cell-

division when this is brought about by the use of what may be termed natural substances —namely, extract of suprarenal gland, creatine, choline, "globin," etc., but it has no effect on artificial substances such as atropine and azur stain. These experiments were repeated many times, and careful controls were carried out simultaneously as described.

The next experiments were made with a view to finding out whether there is any variation in the efficiency of the serum of different persons in stopping cell-division. The serum of six persons was tried in the first instance. The same auxetic jelly was used in each case, and it was found that 0·2 c.c. of serum was sufficient to inhibit the auxetic action completely in four cases. In the other two cases 0·3 c.c. was required. The lymphocytes employed belonged to the blood of the person from whom the serum was taken. Thus :

TABLE I

| Name | Sex | Age | Disease | Serum (c.c.) |
|---|---|---|---|---|
| 1. Ross . . | M | 35 | Healthy . . | ·2 |
| 2. Cropper . . | M | 29 | Healthy . . | ·2 |
| 3. Barlow . . | M | 24 | Healthy . . | ·3 |
| 4. McMahon . | F | 41 | Tonsillitis . . | ·2 |
| 5. Oldham . | F | 17 | Anæmia . . | ·2 |
| 6. Parry . . | M | 21 | Suppurative arthritis | ·3 |

The figures in the last column indicate the amount of serum required completely to inhibit the auxetic action of 0·02 gramme of dried suprarenal extract.

It should be pointed out that in each experiment in the above Table only about a score of

lymphocytes could be examined, and therefore, in order to reduce the error of random sampling, it was considered advisable to repeat the experiments, and to examine the sera of a larger number of people. Only persons who appeared to be healthy were now examined, and the results will be found in Tables II and III.

TABLE II

| Name | Sex | Age | Amount of auxetic (dried suprarenal extract) | Serum (c.c.) | |
|---|---|---|---|---|---|
| | | | | Moderate | Complete |
| 1. Pritchard | F | 14 | 0·02 gramme | ·1 | ·2 |
| 2. Orme | F | 7 | | ·1 | ·2 |
| 3. Fawcett | M | 6 | | ·1 | ·2 |
| 4. Bone | F | 10 | | ·1 | ·2 |
| 5. Peake | F | 7 | 0·025 gramme | ·3 | ·4 |
| 6. Stanton | F | 13 | | ·1 | ·2 |
| 7. Whitehall | F | 11 | | ·1 | ·2 |
| 8. Roach | M | 8 | | ·3 | ·4 |
| 9. Klowski | F | 11 | | ·2 | ·3 |
| 10. Eaton | F | 5 | | ·2 | ·3 |
| 11. Morgan | F | 21 | 0·003 gramme (augmented by 0·0007 gramme of atropine sulphate) | ·2 | ·3 |
| 12. Dickinson | F | 11 | | ·3 | ·4 |
| 13. Brodrick | M | 14 | | ·3 | ·4 |
| 14. Swindell | F | 14 | | —* | ·3 |
| 15. Leahy | F | 15 | | —* | ·4 |
| 16. Griffiths | M | 9 | | —* | ·4 |
| 17. Hudson | F | 7 | | —* | ·4 |
| 18. Miller | M | 14 | | ·2 | ·3 |
| 19. Williams | F | 16 | 0·005 gramme (augmented by 0·001 gramme of choline hydrochloride) | ·1 | ·2 |
| 20. Taylor | F | 6 | | ·2 | ·3 |
| 21. Green | F | 6 | | ·2 | ·3 |
| 22. M'Cann | F | 11 | | ·2 | ·3 |
| 23. Falkner | M | 12 | | —* | ·2 |
| 24. French | M | 13 | | —* | ·2 |

The figures in the last two columns indicate the amount of serum required to inhibit partially and completely the auxetic action.

* In these cases there was only sufficient serum for one test.

TABLE III

| Name | Sex | Age | Serum (c.c.) |
|---|---|---|---|
| 1. Groves | F | 20 | ·3 |
| 2. Wilson | F | 8 | ·2 |
| 3. Rogers | F | 14 | ·3 |
| 4. Friar | M | 12 | ·3 |
| 5. Roney | F | 12 | ·4 |
| 6. Cottam | M | 5 | ·3 |

The figures in the last column indicate an amount of serum which exerted a moderate inhibitory effect. The auxetic jelly employed varied in individual cases as in Table II.

Thus experimentally there appears to be a slight variation of the inhibitory power of different persons' sera on the action of auxetics.

*An investigation of the nature of the "restraining body."*—It has already been stated in a former publication that if the serum is boiled before it is added to the auxetic jelly, or, what amounts to the same thing, if it is added to the jelly before the latter has cooled, its restraining action is destroyed. In other words, the "restraining body" is not thermostable. Hence it seemed to be desirable to find out if this was due to the precipitation of the proteins causing the "restraining body" to be carried down with them. In the first place the whole of the proteins of the serum was precipitated by the addition of excess of alcohol, filtered, and the precipitate redissolved at once in water. It was then dried at 37° C. and dissolved once more in distilled water up to the original volume of the serum. It was found that 0·4 c.c. of this solution

completely inhibited the auxetic action. Not a sign of division could be seen in the lymphocytes, although the auxetic jelly was an excellent one, as shown by the control experiments. The protein-free alcoholic filtrate was also evaporated to dryness, and made up to the original volume as before. 0·4 c.c. of this solution did not affect the cell-division, because all the lymphocytes which were seen were in the act of dividing. The "restraining body," therefore, is precipitated with the proteins, and is not destroyed by alcohol, or, at any rate, by contact with it for a short time. Protein-free serum, on the other hand, does not contain any restraining body. It has already been mentioned that coagulation of the serum by boiling destroys its inhibitory effect on auxetics, and the above result suggests that this is due to the removal of the proteins from the sphere of action.

The next step was to determine if the restraining body attached itself to any particular portion of the protein moiety of the serum. The serum-globulin was separated from the serum-albumin by precipitation of the former by the addition of 15 volumes of distilled water previously acidulated with a few drops of 2-per-cent acetic acid. The clear liquor was decanted off, and the precipitated globulin collected and dissolved in water. It was found that 0·4 c.c. of this solution completely stopped cell-division.

The clear liquor which had been decanted off was then filtered. As this method does

not completely precipitate all the globulin, it contained both albumin and a little globulin. It was evaporated to dryness at 37° C. and dissolved in water to the original volume of the serum. When tested it was found that 0·4 c.c. of this solution also completely stopped cell-division.

Obtained by other methods—viz. serum-globulin precipitated by half saturation with ammonium sulphate and dissolved in water to a strength of 5 per cent., and serum-albumin obtained by saturation with ammonium sulphate and dealt with in the same manner, both the globulin and albumin stopped cell-division completely when 0·4 c.c. of the solutions was used.

On the other hand, a 5-per-cent solution of egg-albumin had not the slightest effect in inhibiting the action of auxetics.

Therefore one may say that the "restraining body," whatever it may be, is attached to the proteins of the serum, and at present I have been unable to separate it from them.

## Conclusions

There is no doubt that serum contains some substance which stops the action of auxetics in inducing cell-division *in vitro*. From the experiments made, it would appear that there is a variation in the strength of this substance in the sera of different persons. Even in healthy persons this variation is apparent. The variation is not great, and although a very large

number of experiments would have to be done to reduce the error of random sampling sufficiently to say definitely in what conditions this variation is most noticeable, I think that the actual fact of its existence is demonstrated.

I have no opinion to offer as to the exact nature of the "restraining body," but undoubtedly it is attached in some way to the proteins of the serum. It does not stop the action of artificial substances, and, as already pointed out (*Induced Cell-Reproduction and Cancer*, p. 253), it probably does not have any direct action on the auxetics, but affects the cells themselves.

## VII

# PRELIMINARY OBSERVATIONS ON THE BEHAVIOUR OF CERTAIN TRYPANOSOMES UNDER THE INFLUENCE OF AUXETICS AND CHANGES OF ENVIRONMENT

By Edward Halford Ross

The *in-vitro* study of blood-cells by the jelly method of examination (1910) has resulted in such considerable advances in our knowledge of their cytology that an attempt has been made to apply the method to the study of trypanosomes. At the Liverpool School of Tropical Medicine observations were made on *Trypanosoma rhodesiense*—a species recently isolated by Stephens and Fantham (1910) as a separate entity; the blood containing the strain—originally found in a man suffering from sleeping-sickness contracted in Rhodesia—was obtained from sub-inoculated rabbits, guinea-pigs, and rats.

At the outset it was found that trypanosomes are very readily killed when placed upon *coefficient* jellies (coefficient jellies are those containing salts and alkali as originally used by H. C. Ross (1909) for the examination of blood-cells). Such jellies produced immediate death of trypanosomes even at the room

temperature—probably because these jellies contain alkali. It was noticed that trypanosomes exhibited the same phenomena at death as do leucocytes and blood-plates—namely, loss of motility, liquefaction of the cytoplasm, and its subsequent diffusion through the cell-wall into the plasma. If the jelly employed contained stain the loss of the trypanosome's motility was accompanied by staining of its granules, and this was succeeded by staining of the so-called nucleus; and then the stain would fade out of the parasite as liquefaction set in. This staining and gradual achromasia renders the observations of trypanosomes simple, for it shows the cell components more readily than the examination of dried, fixed, ordinary stained specimens; but the examination must be rapid, for death, staining, and achromasia follow each other in quick succession on the jelly.

The granules are the first objects to stain and they are the last to fade. With azur stain in the jelly they appear a deep blue. As they take up the dye from the jelly on which the trypanosomes are resting, the motility of the parasite ceases. Then the nucleus and the kinetonucleus stain as death of the parasite occurs. These organs only stain momentarily, for achromasia immediately follows death, and the nuclear staining is the first to fade. But achromasia leaves a sharply defined ring in place of the faded nucleus, and within the ring a well-defined, stained point. After death, if the jelly contains a salt content not exceeding that of normal saline solution, the granules

within the trypanosomes show that peculiar dancing, Brownian movement which is so characteristic of dying protoplasm and which is well seen in leucocytes when, *post-mortem*, their cytoplasm is liquefying. Then the liquid protoplasm begins to diffuse out of the trypanosomes until only their outlines are visible surrounded by the moving granules which have passed out of their interior; the deeply stained ring or nuclear edge with its dark central point, and perhaps the kinetonucleus, remain within the cells embedded in the jelly, until the specimen dries up and the cells become completely disorganised.

The best jellies to show these changes are made as follows: A 2-per-cent solution of agar in water, boiled, filtered, and sterilised. To 5 c.c. of this mixture add 1 c.c. of a 2-per-cent solution of azur II, and make up the mixture to 10 c.c. in a test-tube with normal saline solution. Pour a little of this molten jelly on to a microscope slide and allow it to set and cool in a thin film. Place a drop of the citrated blood containing the trypanosomes on to a cover-glass and invert it on to the jelly—the blood spreads out evenly between the cover-glass and the jelly. The trypanosomes remain motile for a few moments only, and examination under the microscope must be immediate. The specimen should not be incubated.

But if such a jelly is made with water instead of the normal saline solution the majority of the trypanosomes will be found to have altered

their characteristic shape entirely, to have become round, and, in many instances, to have lost their "tails." In fact, they will be found to resemble the "latent bodies" discovered by Moore and Breinl (1907) in the internal organs of animals suffering from trypanosomiasis.

It was found that if the jelly contained such a salt-content so as to keep the erythrocytes at the verge of hæmolysis, and if it also contained one of those substances which have been called auxetics—substances which have been shown by H. C. Ross (1910) to induce cell-division and reproduction—the same result occurred, namely, the production of the rounded body. A useful jelly to employ for this purpose is that which has been utilised to induce divisions of red blood-corpuscles in anæmic blood (E. H. Ross, 1911). This result is invariable and can be produced by the use of auxetics such as creatin, "globin," theobromine, and putrid extract of suprarenal gland, or the extracts of dead tissues. It would seem probable therefore that the addition of water to blood lakes the erythrocytes and produces a liberation of hæmoglobin into the plasma. This substance is known to be an auxetic and to induce divisions in blood-corpuscles. The round forms of trypanosomes so produced show, when stained by the jelly method, the ring-shaped nucleus with its central deeply coloured point, and in another part of the parasite the kinetonucleus, the granules, and sometimes the remains of the chromatin-edged

flagellum and undulating membrane. Death in these cells is almost immediate on the jelly, and it is followed by the phenomena already described. The " latent bodies " show but little, if any, increased resistance to the killing action of such jelly.

The process of conversion of trypanosomes as commonly found in the peripheral blood into the round forms may be actually watched by placing a drop of blood from an infected animal, showing trypanosomes in the peripheral circulation, on to a jelly which is neutral and which contains no stain and only such a content of salts as will keep the erythrocytes at the verge of hæmolysis ; and then examining the specimen immediately under the microscope at the room temperature. Such a jelly will lake some of the red corpuscles, for, as has already been shown (E. H. Ross, 1911), their vitality on such jellies varies within certain limits ; and the consequent liberation of auxetic will result in the " head " of the parasites swelling slightly and the " tails " or flagella becoming foreshortened until they shrink into the trypanosome when the parasite becomes a round, motionless cell. Sometimes the tip of the tail becomes rounded also, in which case the " bulb " falls off or is absorbed into the general body-mass of the parasite. As soon as the flagella of the trypanosomes have completely disappeared motility is arrested ; but until this occurs the parasites remain wriggling and vibrating. Ultimately death ensues with the extravasation of the cell contents.

It has been suggested that this formation of the round body is due to some physical effect of the water, that it is due to some alteration of the surface tension. But Miss Muriel Robertson (1911) has stated that water when added to trypanosomes will produce their division, and she has been able to watch this division hours after the water—in small quantities—has been added to the blood. In this case the round bodies were not formed ; whereas if auxetics are employed in the jelly they are produced. But water—in larger quantities—liberates auxetic and then the round bodies are formed at once.

While attempting to exclude the possibility of this round-body formation being an act of degeneration on the part of the trypanosomes a series of jellies were employed which contained various contents of stains, salts, alkali, and water. To this end living trypanosomes were placed on a jelly which had not been rendered alkaline in the ordinary way for blood-cell examination, and which, therefore, was slightly acid, as the solutions of agar are unless neutralised. This jelly contained only salts sufficient to prevent complete hæmolysis—it produced laking of many of the erythrocytes, but not all of them, for complete destruction of the blood causes destruction of the parasites also—and apparent conjugation of some of the trypanosomes was seen in specimens examined immediately ; two of the round bodies appeared to fuse together to form one cell. When these slightly acid watery jellies were used two of

the parasites would appear to fuse together to form one wriggling mass; it seems a different process to the usual agglomeration of trypanosomes so commonly seen in wet specimens. The fusion occurred while the round shape was being formed and before the tails had completely disappeared; then the parasite became immobile and the tails were lost in the single cell mass.

An attempt was made to obtain permanent specimens of these fused cells. By a method already described (Cropper, 1911) the cover-glass was removed from the surface of the jelly and the blood on the jelly was transferred to a clean slide, and subsequently fixed and stained. The parasites were found to be in various shapes and sizes and the fused cells were stained united, for no line of demarcation could be discovered between the joined cells. In the same specimen there were also found oval bodies the result of this fusion; for they contained double nuclei embedded in one trypanosome protoplasm. Sometimes a trypanosome containing two nuclei would fuse with one having a single nucleus; in this case the resulting body would contain three nuclei and perhaps two or three kinetonuclei. After fusion the chromatin collects into rods and streaks, while the kinetonuclei remain distinct in different parts of the cell. The vacuoles usually remained distinct also. This stage was reached within half an hour on the jelly, when the cells died and no further development occurred; so with their morphology in

the fixed specimens. Later changes under the influence of auxetics have not yet been seen, as the jelly soon kills the parasites when they are pressed down by the cover-glass into the agar.

But it was found that the process of removing the cover-glass from the jelly caused tearing and distortion of the cells. Another simpler method was sought. Some citrated blood containing trypanosomes was dropped on to a slide in the form of a ring. Into the dry centre of the ring a drop of distilled water was placed, and the water allowed to impinge on, and to mix with, the still wet ring of blood at one point; the water slowly diffused through the blood. Then a minute drop of a 4-per-cent solution of citric acid was mixed with the water in the centre of the ring and the slide allowed to dry slowly at the room temperature, and subsequently fixed and stained. Where the watery solution of citric acid first touches the blood hæmolysis occurs. This liberates hæmoglobin which is carried on through that part of the ring of blood where the water mixes with the plasma. After fixing and staining the dried specimen so made, trypanosomes were found in all stages of becoming converted into round forms, and several in the act of fusion. This process is a very simple one, and it gives clear and beautiful pictures demonstrating the behaviour of the living cells on the jelly. Where the water and the liberated hæmoglobin have mixed with the blood and have diluted it without laking all the corpuscles,

the latent bodies are found; nearer the acid solution the act of fusion is seen; and in the centre of the ring, where the acid solution is more concentrated, all the blood-cells and trypanosomes are destroyed, and there is a blank.

Moore and Breinl found the round forms in the internal organs of infected animals, namely, the lungs and spleen. It would seem possible therefore that there is always more auxetic circulating in the blood of those organs derived from the hæmoglobin from degenerate blood-cells and possibly other substances derived from the death of other cells; it is known that creatine and tyrosine are auxetics, and perhaps their presence will account for the discovery of the round forms of trypanosomes in the internal organs in ordinary infections in vertebrates. Or perhaps some alteration of the plasma in the internal organs brings the parasites more under the influence of auxetics in the lungs, spleen, etc.; the round forms can be produced on a slide by simply altering the density of the blood or by lowering its salt-content—but this always results in the liberation of hæmoglobin. Dividing blood-corpuscles are rarely, if ever, seen in the peripheral circulation of mammals; indeed, as Cropper (*loc. cit.* p. 80) has shown, the serum of the peripheral blood will prevent the action of auxetics. Extracts of dead tissues, on the other hand, actually contain auxetics. The round-body formation is hindered also by serum; it can be produced by diluting the

serum. But it is not an act of degeneration, for, as Fantham (1911) has demonstrated, the round bodies can be induced to become flagellates by placing them in fresh, uninfected blood from the peripheral circulation. The round-body formation and their fusion therefore would seem to be brought about by an alteration in the environment of the parasite—an alteration of environment which is accompanied by the liberation of auxetic. Replace the round forms in serum unaltered and the trypanosomes regain their characteristic shape. This alteration of environment and its action on blood parasites appear to be of great importance. If blood containing malaria parasites is mixed with water the gametes are produced ; if blood containing *Leishmania* is placed in an acid medium containing hæmoglobin (auxetic) flagellation occurs as shown by Leonard Rogers ; and now, if blood containing trypanosomes is exposed to auxetics the rounded bodies are formed and apparent fusion takes place.

Sir Ronald Ross and Dr. David Thomson (1911) have described peculiar appearances in the large mononuclear leucocytes found in the peripheral blood in cases of sleeping-sickness ; these consist of vacuoles and the presence of " red-staining debris." Similar appearances can be seen in any mammal infected with *Trypanosoma rhodesiense*. It will be remembered that Schaudinn believed that leucocytes played some part in the life-history of trypanosomes. A rabbit recently inoculated with this try-

panosome was therefore examined by the jelly method every day after inoculation. On the third day after inoculation the peripheral blood showed, on a stain-containing watery jelly, round bodies in the larger lymphocytes which took up the stain quickly before the granules of the cell; and these bodies exhibited a *coefficient of diffusion* (H. C. Ross, 1909) similar to the trypanosomes; they also possessed a nucleus and a dot which, however, immediately became achromatic. As soon as the lymphocytes themselves stained, these bodies became obscured by the staining granules of the leucocytes. But fixed and stained specimens show only the "debris" described by Ross and Thomson.

The acid method of liberating the auxetic in the blood was repeated, but the specimen dried more slowly at a temperature of 86° F. before fixing and staining. It was found that several of the larger lymphocytes had taken up unattached latent bodies, and that these were stained *in situ*. Here they showed the red nucleus and the kinetonucleus, and when stained by Leishman's stain the resemblance between them and *Leishmania* was noted. The possibility of this being an act of phagocytosis has been suggested; but it only occurred in the lymphocytes. The other blood-cells remained free.

In a recent publication Warrington Yorke (1911) has described a trypanosome keratitis in goats, the infected blood extravasating into the cornea. But he pictures therein bodies

closely resembling the fused trypanosomes as seen by the method described above. His drawings, in several examples, follow faithfully those of the result of the anastomosis of two latent bodies produced by the liberation of auxetics—a liberation known to occur after injury, inflammation, and cell-death. As already mentioned, Miss Muriel Robertson (1911) claims to have induced divisions of trypanosomes by adding water to the blood containing them and watching them for hours; this may be the result of prolonged action of the water in causing hæmolysis, cell-death, and the production of auxetic. But the acid method described in this chapter produces the phenomena mentioned as fusion, and at a higher temperature engulfing by lymphocytes, in less than half an hour; divisions of the trypanosomes were not noticed in the shorter period.

Lastly, I have seen appearances in the lymphocytes resembling those described by Ross and Thomson in cases of endemic cirrhosis of the liver accompanied by splenomegaly occurring in Egypt, Arabia, and Syria. And in this connection it is interesting that Nicolle (1908) found *Leishmania infantum* in cases of splenomegaly among the children of Tunis; while Critien (1911) has discovered the same thing in some children in Malta. Hartman (1909) has described *Leishmania*-like bodies within the endothelial cells of the lungs in human infections with *T.* (*Schizotrypanum*) *cruzi*.

Professor Minchin (1911) has described re-

cently an intracellular stage in the development of *T. lewisi* in the epithelial cells of the midgut of the flea. He states: "The earliest change which such a trypanosome undergoes is that the body assumes a bulbous form. . . ." He describes how it becomes spherical inside the epithelial cell, and then "block-like" until a number of daughter trypanosomes are produced by a process of multiple fission; the daughter trypanosomes are discharged from the epithelial cell. In the grumous blood commonly found within the stomach of a flea or fly there must be a considerable amount of free auxetic. There is reason to believe that molecular death liberates auxetic within leucocytes, and perhaps the same occurs in epithelial cells; this would account for the intracellular development of trypanosomes. There is an interesting analogy in the development of *Lymphocytozoon cabayæ* into spirochetes.

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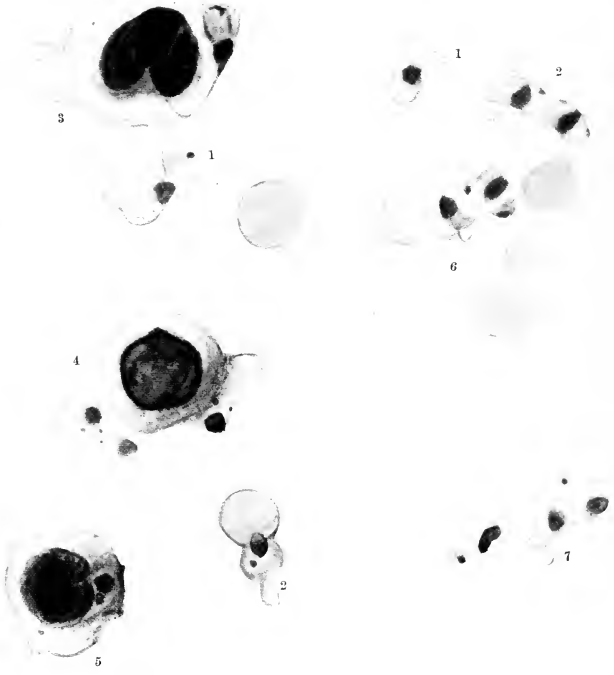



THE BEHAVIOUR OF CERTAIN TRYPANOSOMES UNDER THE INFLUENCE OF AUXETICS.

1. *Trypanosoma rhodesiense*, becoming rounded; first action of auxetic.
2. Rounded forms; early action of auxetic.
3, 4, and 5. Further action of auxetic at higher temperature.
6. Fusion: later action of auxetic.
7. Result of fusion.

# VIII

## THE DEVELOPMENT OF A LEUCOCYTOZOON OF GUINEA-PIGS[1]

By Edward Halford Ross

The presence of "bodies" within the large mononuclear leucocytes of guinea-pigs was first noticed by Kurloff (1898). He described them as inclusions; for in a drop of guinea-pig's blood he noted that many of the large lymphocytes contained, within their cytoplasm, clear, spherical vacuoles which were distinct from the nucleus, and which had not been described before; and he suggested the possibility of these bodies being accessory nuclei. Since their discovery by Kurloff they have been subjected to much research; and papers describing various observations concerning them have been published by Burnett (1904), Staubli (1905), Goldhorn (1905), Ledingham (1906), Howard (1907), Pappenheim (1908), Patella (1908), Hunter (1909), and Schilling (1911).

Kurloff noticed that when the blood containing these bodies was fixed and stained, they contained a nucleus-like structure staining

[1] A paper read before the Royal Society on February 29, 1912, and reprinted by permission.

with nuclear dyes, but he believed them to be vacuoles formed by a secretion-product of the cells which held them. Ehrlich (1906) also thought that Kurloff's bodies represented some "Secretstoff." Dr. Ledingham, to whom I am indebted for much information, seems to have been the first to suggest the possibility of their parasitic nature, and he mooted an analogy to the *Cytorryctes variolæ* or *vaccinæ*. Goldhorn (1905) boldly called them leucocytozoa. The most recent work published on the subject is that of Schilling (1911). He has examined these bodies by "vital" staining with azur, and he has described some of the earlier stages of their development while in the mononuclear leucocytes (lymphocytes). He believes that the rod stage precedes the granule stages, and this has caused him to adhere to the opinion that Kurloff's bodies must be classed with the Chlamydozoa, symbiotic structures, or vaccine inclusions.

Early in 1911, while examining a guinea-pig's blood by a new jelly method of examination of blood-cells, H. C. Ross saw Kurloff's bodies, and pointed out to me that the method demonstrated the probability of their parasitic nature. The new method, which was devised partly at the suggestion of Sir Ronald Ross, K.C.B., has already been fully described (H. C. Ross, 1909); the bodies then seen were in the earlier stages of their development. But the inclusions stood out so clearly by this method that I determined to continue the observations, for this technique seemed to show details of

structure which had not been described before ; and since by the new process the bodies can be subjected to the action of various stains and chemical agents there was a possibility of the phases of their development being observed. I may state that I have now been able to convince myself that these bodies are living parasites of the mononuclear white corpuscles (lymphocytes), and henceforth in this chapter I propose to call them such.

I use a modification of the original jelly method—it is as follows : A 2-per-cent solution of agar in water is boiled, sterilised, and filtered. To 5 c.c. of the filtrate is added 0·5 c.c. of a 10-per cent solution of sodium chloride in water, and 0·5 c.c. of a 1-per-cent solution of azur II in water. The total bulk of the mixture is made up to 10 c.c. in a test-tube. When molten, a small quantity of the jelly is allowed to spread itself in a thin film on a microscope slide and to cool and set. Then a drop of guinea-pig's blood (or citrated blood) containing Kurloff's bodies (about 90 per cent of the guinea-pigs examined by me, which were obtained from dealers in England, are infected) is placed upon a cover-glass, and this is inverted on to the set jelly. The blood spreads out between the cover-glass and the surface of the jelly, and, after an interval of five minutes, during which the blood-corpuscles come to rest, the specimen may be examined under the higher powers of the microscope. After a further interval of a few minutes—the exact period varying

slightly with the temperature of the room—the granules of the leucocytes begin to stain, after which their nuclei gradually stain a deep blue; the contours of the erythrocytes, as well as those of the leucocytes, show up clearly, and the method is a pretty example of *in-vitro* staining. In some of the larger mononuclear cells the colourless parasites will be noticed at one side of the protoplasm. These parasites are inside the cell, because the shape of the nucleus of the lymphocytes is moulded according to the size of the parasite, which grows larger as it develops—in its youngest stages it is small, while in its last intracellular stages it bulges the lymphocyte cell-wall and squeezes the nucleus into a small space; this point is of interest because, as Hunter has shown, Patella claimed that Kurloff's bodies lie upon and not in the lymphocytes. In cells containing the larger parasites smaller vacuoles can also be seen; these latter always remain clear and transparent even when examined on stain-containing jellies, and they vary in numbers, and slightly in size, in different examples. It has been suggested that these smaller, subsidiary vacuoles are polar bodies, but more probably they contain excretory products of the lymphocytozoa into the cytoplasm of their hosts, for they become larger and more numerous as the parasite grows.

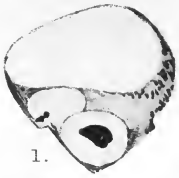
When examined on the jelly, and immediately before the staining of the nucleus of the leucocytes, the contents of the parasites begin

to stain [1]—the internal chromatin structure of the spherical sac embedded in the lymphocytes' cytoplasm becomes purple and remains stained for several hours, so that its examination is readily made. If the bloods of a number of infected guinea-pigs are watched in this manner from day to day, what appear to be the successive stages of the growth of the parasite in the lymphocyte can be seen and drawn; but the leucocytes of a single animal at any particular moment contain, usually but not always, parasites in the same stage of development. The cycle, however, can be followed by observing the blood of one guinea-pig hourly.

The interpretation which I place upon the appearances I have seen is as follows: The parasite presents itself, in the smallest phase of its intracorpuscular cycle, as a tiny translucent body embedded within the cytoplasm of the larger mononuclear blood-corpuscles and near the periphery of those cells. Usually one of such bodies is present in any one cell, but occasionally two or even three parasites may occur in the same cell. The parasite, in this early stage, contains a double purple

[1] It must be emphasised that if the jelly contains excess of salts or impure stains, the wall of the parasite will stain in an irregular manner, and then patches of stain will hide its contents. Furthermore, if the blood on the jelly dries, or if the blood is fixed in any way, the same thing occurs. Similarly, patchy staining is obtained by the various fixed-film methods in vogue, as, for example, Romanowsky's or Jenner's stains. Even azur stain, when applied to the dried or fixed films of blood, will not demonstrate the details of the development of the parasite. No alkali should be added to the jelly.

dot (figs. 1, 2); in this phase it resembles the Leishman-Donovan bodies found in human leucocytes in cases of Kala Azar. When first seen the dot is motionless, but after a time on the jelly, as the lymphocyte host becomes disorganised, it may show some Brownian movement. In the next stage the parasite is larger, and the chromatin dot has divided into two or more dots until the sphere-like sac may be packed with them (fig. 3). Then each dot becomes dumbbell-shaped (fig. 4), and again, by a simple process of elongation, rod-shaped (figs. 5, 6, 7). The parasite may contain one of these rods (fig. 9), or it may be full of them—the actual numbers varying in different examples. Sometimes a parasite may contain one or more rods, some dumbbells, and some dots. But the size of the parasites increases steadily with these successive stages of the development of their contained chromatin (compare figs. 1 and 15). During the rod-formation the smaller subsidiary vacuoles already mentioned appear in the cytoplasm of the host-cell (figs. 3, 5, 12); they never contain any chromatin and remain unstained. With its growth the parasite begins to compress the nucleus of the lymphocyte (figs. 13, 14), and the wall of the latter can be seen as a shell enclosing the parasite (figs. 14, 15, 16). The rods grow longer and thicker (figs. 8, 9, 10) until they stretch across the parasite, and their ends may be doubled against its wall, and they may then present in optical section an erroneous impression of flattening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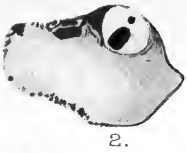
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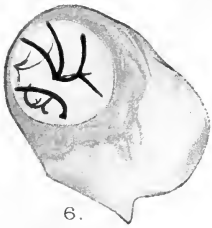
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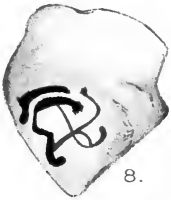
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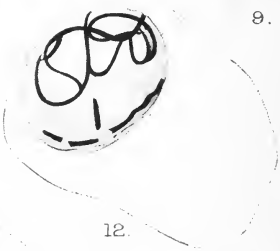
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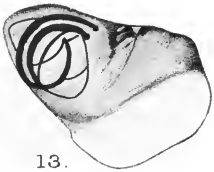
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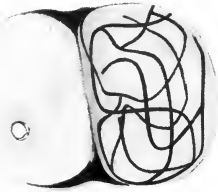
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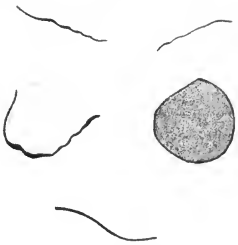
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DEVELOPMENT OF A LEUCOCYTOZOON OF GUINEA-PIGS.

or a terminal bulging (figs. 8, 13, 14). In the next stage a stout flagellum grows out from both ends of the rod (figs. 8, 11, 12, 13), which becomes rolled up in a coil within the sphere (figs. 13, 14, 15). The rod with its two flagella splits longitudinally in its whole length (figs. 8, 12), and this process of splitting takes place again and again. The fission throughout is always lengthwise, never transverse. A specimen in this stage will show the parasite, now equal in size to the original dimensions of its host-cell, bulging the wall of the latter, compressing the nucleus into a small space, and containing within its interior a mass of woven, twisted, and intertwined purple threads, a conglomerate maze of worm-like spirilla stained red by the azur dye (figs. 15, 16).

Arrived at its maturity, the parasite breaks away from the shell of its host-cell and then bursts, setting free the threads into the plasma (fig. 17). But the flagellate forms, owing to the fact that they are stained, are dead and motionless, and they may remain attached to the shrunken sphere sac, their ends waving in the currents set up.

It was found very difficult to demonstrate the motile, flagellate forms of the parasite when free in the blood. They cannot be seen then by the jelly method, because, probably, they stain momentarily as the trypanosomes do, and immediately die and become achromatic, and unless stained they are not visible. By the examination of ordinary wet films of the blood I was unable to demonstrate the presence

of these free flagella, although a disturbance of the corpuscles was frequently seen. But the blood of some infected guinea-pigs, drawn under all aseptic precautions and examined by the dark-ground illumination, showed free-swimming spirochæte-like bodies. It was not until the blood of highly infected guinea-pigs containing fully matured lymphocytozoa was treated with an equal part of a 1-per-cent solution of "globin."[1] and incubated at 37° C. for eight hours that the free flagellate forms in the blood-plasma could be fixed and stained by ordinary methods (fig. 18). Even by this process it is not always possible to demonstrate them, and the maceration involved gives them the appearance of spirilla with blunt ends. However, some of the spirilla obtained after the treatment with the "globin" show the wavy outline of spirochætes. Sir Ronald Ross was the first to suggest that these flagellate forms constitute the gametes of the parasite; this seems quite probable, though no separate female form has yet been noticed. It will be remembered that Lewis suggested that trypanosomes are sperms, and, perhaps, these spirochæte-like bodies are similar stages of a larger parasite.

What may possibly be the last phase of this parasite has occasionally been seen in preparations which had been submitted to the action of "globin" for a further period of

[1] The filtrate of a solution of hæmoglobin which has been decomposed by heat. H. C. Ross claims that this substance induces the division of certain cells.

four hours. It is an object which resembles somewhat the trypanosome "latent bodies" described by Moore and Breinl (fig. 19). Hunter has also mentioned the presence of amœboid forms of this parasite being free in the plasma, but he does not picture them. These may be the form now drawn (fig. 19).

Dr. J. W. Cropper and I have repeated and can confirm the experiments of Ledingham (1906) and Hunter (1909)—namely, that newly born guinea-pigs do not show these lymphocytozoa in their blood. Although a pregnant animal may be markedly infected, the young, when born, possess no parasites. As has been already observed by these writers and by Schilling (1911), the number of parasites found in both the peripheral blood and in that of the internal organs of any one infected guinea-pig varies greatly from day to day. The parasites seem to appear in large numbers, to diminish, to disappear, and then, after a varying period of time, to reappear. Except for a slight anæmia, shown by the presence of an increased number of erythroblasts in the peripheral blood, the guinea-pigs do not suffer apparently. The livers of many of these infected animals show, however, single or multiple white patches of necrosis varying in size between that of a pin's head to that of a large pea and extending into the substance of the organ. But we have no proof, as yet, of their direct relation to the parasite.

Fixed specimens of the various stages of the development of this parasite may be made

by substituting an equal amount of a 1-per-cent solution of caustic soda in the jelly for the sodium chloride solution. By this means the red blood-corpuscles are laked, but the nuclei of the leucocytes and the chromatin of the lymphocytozoa stain well. The cover-glass can then be lifted from the jelly and mounted while still wet in Canada balsam. Many of the leucocytes with the contained parasites will adhere to the cover-glass and will retain their stain.

Since this paper was written, Hindle has published a preliminary note (Hindle, 1911) "On the Life-cycle of *Spirochæta gallinarum*." He asserts that these spirochætes possess an intracellular stage within the cells of the Malpighian tubes of the tick, *Argas persicus*. In view of the life-history of this lymphocytozoon of guinea-pigs his work is of great interest.

I have to express my indebtedness to Dr. J. W. Cropper and to Dr. H. Bayon for their help in these researches; the latter was the first to recognise the free-swimming, spirochæte-like bodies. I also wish to thank Professor Minchin for much help and advice and the interest he has taken in this work.

## *Summary*

Kurloff's bodies are parasites, lymphocytozoa inhabiting only the mononuclear cells of the guinea-pig's blood.

These lymphocytozoa have an intracorpuscular stage, and ultimately give rise to free-swimming, spirochæte-like bodies, which may be gametes.

The development of the spirochæte-like body is demonstrated.

The name *Lymphocytozoon cobayæ* is suggested for this parasite.

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## NOTE

Since the foregoing paper was sent to press in this volume, I have received information from Dr. John Thomson, of the Liverpool School of Tropical Medicine, that he had discovered spirochætes in the fæces of some guinea-pigs at Liverpool. As it is possible that these spirochætes may have a relation to those spirochæte-like bodies developed from Kurloff's bodies, I have asked him to write a short account of his work.

In the penultimate paragraph of Dr. Thomson's description of his spirochætes, he states that he has seen similar bodies in the fæces of wild rats. This is a very interesting observation, for Dr. Andrew Balfour (*Fourth Report of the Wellcome Tropical Laboratories*, Gordon College, Khartoum, p. 123) states that he has seen structures resembling Kurloff's bodies in the blood of rats. Balfour (*ibid.*) has also found similar structures in the mononuclear cells of the blood of fowls, where spirochætes are frequent. He

writes: "Indeed, I have seen it suggested that the intracorpuscular stage of fowl spirochætes, which I have described in red cells, was in reality of the nature of plasmosomes or Kurloff's bodies, hence the importance of bearing them in mind."

E. H. R.

## AN INTESTINAL SPIROCHÆTE OF GUINEA-PIGS

By John G. Thomson, M.A., M.B., Ch.B. (Sir Edwin Durning-Lawrence Research Scholar, Liverpool School of Tropical Medicine).

For some time I have been examining the fæces of guinea-pigs, and have been much surprised to find that, on certain days, spirochætes occurred in large numbers.

The animals I first examined were inoculated with *Trypanosoma rhodesiense*, and as spirochætes occurred in the fæces of many of these infected guinea-pigs the possibility of some relation suggested itself; I therefore examined some normal animals. But if a sufficient number of normal guinea-pigs are examined, spirochætes are found in them also. It is noteworthy, however, that Balfour has found spirochætes in the intestinal ulcers of certain animals infected with trypanosomiasis in the Soudan; and we are acquainted with *S. dentium*. Spirochætes have also been described in certain forms of diarrhœa. But I cannot find any reference to the spirochætes of the intestines of guinea-pigs in the literature.

I am uncertain whether the spirochætes found in the intestinal contents of normal guinea-

pigs are pathogenic or not. But it is quite possible that they do become pathogenic when the animal's resistance is lowered. I find, for example, that the spirochætes in the fæces of uninoculated animals are certainly not nearly so numerous nor so frequently found as those in the fæces of guinea-pigs suffering from diseases such as trypanosomiasis; again, I found that guinea-pigs which had died of tuberculosis had swarms of spirochætes in the contents of the large intestine, though no ulceration was found there.

The technique for the examination of these spirochætes is very simple. It is possible to tell, within five minutes, whether the spirochætes are present by employing the following method: The intestinal contents is mixed with saline solution, and a drop of the mixture transferred to a clean slide. It is spread out thin and allowed to dry and is fixed in the flame. Stain with a solution of methyl or gentian violet (1 drop of a saturated alcoholic solution to 1 c.c. of tap-water). Heat gently while staining for a minute, and then wash.

The spirochætes are very small—about 3 to 5·75 $\mu$ from end to end, *i.e.* measuring in a straight line and ignoring the curves. They are pointed at both ends, and show darker areas arranged at intervals along the body. The curves are very regular, and vary from two to three undulations. I have counted as many as six darkly stained points in one spirochæte. Some of these spirochætes are as long as 10 $\mu$, but these are evidently double forms end to end,

or possibly dividing forms. Examined under the dark-ground illumination, they are seen to proceed with a corkscrew movement, the screw being right-handed.

I am unable to state the mode of infection of the alimentary canal with these parasites, and, so far, I have only found them in the large intestine. It is possible that the infection is by the mouth. But they occur in apparently healthy guinea-pigs.

I have also seen spirochætes in the fæces of wild rats, and these seem to be a little larger than those found in the guinea-pig.

I wish to thank Dr. J. A. Sinton for his assistance in studying these spirochætes.

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